# "EVALUATION OF THE MICROBIOME IN PERIODONTAL HEALTH AND PLAQUE COVERING SUPRAGINGIVAL CALCULUS USING NEXT GENERATION SEQUENCING TECHNOLOGY"

*Dissertation submitted to*

**THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY**

*In partial fulfillment for the Degree of*

**MASTER OF DENTAL SURGERY**

**BRANCH II**

**PERIODONTOLOGY**

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# THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY

# CHENNAI

## DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"EVALUATION OF THE MICROBIOME IN PERIODONTAL HEALTH AND PLAQUE COVERING SUPRAGINGIVAL CALCULUS USING NEXT GENERATION SEQUENCING TECHNOLOGY"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. Swarna Alamelu, M.D.S.,** Reader, Department of Periodontology, Ragas Dental College and Hospital, Chennai.

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# CERTIFICATE

This is to certify that this dissertation titled **"EVALUATION OF THE MICROBIOME IN PERIODONTAL HEALTH AND PLAQUE COVERING SUPRAGINGIVAL CALCULUS USING NEXT GENERATION SEQUENCING TECHNOLOGY"** is a bonafide record of work done by **Dr. K. Santhosh Kumar** under my guidance during the study period 2016-2019.

This dissertation is submitted to **THE TAMILNADU Dr. MGR MEDICAL UNIVERSITY** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY, BRANCH II- PERIODONTOLOGY.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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# CONTENTS

# LIST OF ABBREVATIONS

| ABBREVATION | EXPANSION |
|---|---|
| PSD | Polymicrobial Synergy and Dysbiosis |
| DNA | Deoxyribonucleic acid |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal Ribonucleic acid |
| NGS | Next Generation Sequencing |
| PCR | Polymerase Chain Reaction |
| RT-PCR | Real Time Polymerase Chain Reaction |
| CVE | Crystal -Violet Erythromycin |
| PI | Peri-Implantitis |
| HOMINGS | Human Oral Microbiome Identification Next Generation Sequencing |
| HOMD | Human Oral Microbiome Database |
| QIIME | Quantitative Insights Into Microbial Ecology |
| OTU | Operational Taxonomic Unit |

# LIST OF TABLES

# LIST OF GRAPHS

# LIST OF FIGURES

# *Introduction*

# INTRODUCTION

Periodontal disease is a microbially driven chronic inflammatory condition which affects the tooth supporting structures resulting in destruction of connective tissue, alveolar bone resorption and eventually tooth loss. Although the plaque associated biofilm initiates the disease process, it is the host immune inflammatory response that inflicts the irreversible tissue damage[26]. The role of plaque in the initiation of periodontal disease was successfully demonstrated by Loe's "Experimental Gingivitis Model" and since then various hypotheses have been put forth to explain the role of the plaque microbiota in the etiology of periodontal disease[80, 145, 36].

The current understanding is that periodontal disease develops as a result of major disruptions in the ecological balance of the oral microbiota as a result of the environmental changes in the oral cavity, which is termed as dysbiosis[45]. The complex equilibrium between the resident organisms in the niche is responsible for the maintenance of a healthy state and when the diversity and the relative proportion of taxa is disturbed, a dysbiotic state emerges leading to the formation of a disease- associated microbiome[63, 18].The composition of the oral microbiome varies from one individual to another and from one ecological niche to another, which is in part due to the host immuno-inflammatory response. Consequently, it becomes important to understand the genomic composition of the oral microbial community and the factors that

influence this ecological balance to prevent and keep a check on the progression of disease[63].

Dental calculus is a complex, mineralized bacterial biofilm that forms from dental plaque on the surfaces of teeth and is composed of crystals of various calcium phosphates[138]. It develops coronal or apical to the gingival margin as moderately hard deposits and is always covered with vital, non-mineralized biofilm [152]. The formation of calculus varies widely among population, the rate of which is known to be affected by several factors – both endogenous and exogenous viz. salivary flow rate, presence of salivary mineral content, dietary patterns, oral hygiene practices and genetic predisposition[58].

Dental calculus is considered as a primary predisposing and plaque retentive factor in the development of periodontal disease. It plays a key role in maintaining and accentuating periodontal disease progression by keeping the plaque biofilm in close contact with the tooth surface and gingival tissues leading to various pathological changes[67]. It is difficult to separate the effects of plaque and calculus on the gingival tissue, because calculus is always covered with a non-mineralized layer of plaque. Various cross-sectional and longitudinal studies have shown the association of calculus and periodontitis[85, 59, 20].Increased loss of attachment associated with substantial amount of plaque and calculus seems to be the prevalent disease phenotype in the developing/less developed countries[156, 59].

A myriad of conventional methods hasbeen used to analyze the composition of the periodontal microbiome which includes microscopy, cultural analysis, enzymatic and immunoassays[157]. However, many bacteria are fastidious, slow growing and require complex growth media and other specific requirements. All these could have led to a biased understanding of the microbial etiology as the disease could be attributed to those species that happen to thrive under such conditions while the others remain undetected[122].

The advent of culture independent open-ended methods, has greatly improved the identification of microorganisms, many of which could not be either cultured or detected earlier. The open-ended methods include the Sanger/Pyrosequencing of the 16s rRNA gene, which is widely conserved across all organisms. They can be extracted, amplified, sequenced and compared with databases such as Human Oral Microbiome Database (HOMD). But these methods were expensive, laborious and time consuming[93].

The use of Next Generation Sequencing (NGS) technology for the 16s rRNA gene has led to the generation of an increased sample throughput with up to 27 million sequences being generated in a single run. The simplicity and affordability of NGS has led to an enormous generation of data and has greatly expanded our knowledge of the oral microbiome[159]. Various metagenomic studies have been carried out in different types of periodontal and peri implant conditions and have demonstrated different microbial profiles across ethnic and geographic regions[54, 3, 125].

Previous studies have been done in our department to evaluate the microbiome of the plaque biofilm in various periodontal conditions viz. gingivitis, chronic periodontitis with either pockets or with gingival recession using NGS technology. (Unpublished data). But the microbial profile of plaque that is present and covering the supragingival calculus has not been studied yet. This study hypothesizes that the microbial profile present on the plaque covering the calculus might have an influence on the formation of subgingival microbiota that may play a role in the transition of gingivitis to periodontitis. The aim was to evaluate the microbial profile of the plaque biofilm in patients who presented with a moderate to severe calculus supragingivally and to compare it with that of the microbial profile of the plaque in periodontal health.

# *Aim & Objectives*

# AIM AND OBJECTIVES

- To evaluate the microbiome from supragingival plaque samples of periodontally healthy subjects and patients with plaque covering supragingival calculus using NGS technology.

- To compare the microbiome of plaque covering supragingival calculus with that of periodontal health.

# *Review of Literature*

# REVIEW OF LITERATURE

## ORAL MICROBIOME:

Oral microbiome, by definition, is the collective genome of microorganisms that reside in the oral cavity. Many researchers believe that the characterization of oral microbiome is a vital step in understanding oral health and systemic diseases. The oral cavity has densely populated microbial communities and has the largest number of commonly shared microbes among unrelated individuals. As such, oral microbiome provides an ideal source for biomarker discoveries due to low inter- and intra- biological variations, in contrast to other biomarkers originating from the host. The oral cavity and associated nasopharyngeal regions are also an ideal environment for the growth of micro-organisms.

**Joshua Lederberg in (2001)** coined the term 'Microbiome'. It is defined as the totality of micro-organisms and their collective genetic material that is present in the human body[86]. This term has been chosen by the Human Microbiome Project and referred as favoured nomenclature to describe complex oral bacterial community, their genetic elements and environmental interactions which may be involved in the disease process[28].

The Pioneering work on the study of the diversity of the human microbiome was carried out by **Antonie van Leewenhook**[30,31]who as early as

the 1680s had compared the oral and faecal microbiota. He found that there was a stark contrast in the microbiota between these 2 habitats and also from individuals who were in states of health and disease. Thus, the discovery of the profound differences in the microbiota at different sites in the body and at different states of health is as old as microbiology itself. The present-day technologies help us gain more insight into the reasons behind such differences and also understand as to how these transformations from one state to another can be regulated.

The human microbiome was classified by **Turnbaugh et al (2007)**[147] into 2 types as core microbiome and a variable microbiome. The 'core' microbiome is shared by all individuals and comprises of the predominant species that exist at different places in the body under healthy conditions. The'variable' microbiome is unique to the individual and has evolved in response to unique lifestyles and phenotypic and genotypic determinants.

**Paster et al (2001)** [112] stated that the organisms identified only from diseased sites deserve further study as potential pathogens. Based on the sequence data in this study **(**Bacterial diversity in human subgingival plaque), the predominant subgingival microbial community consisted of 347 species or phylotypes that fall into 9 bacterial phyla. Based on the 347 species seen in his sample of 2,522 clones, he estimated that there are 68 additional unseen species, for a total estimate of 415 species in the subgingival plaque. When organisms found on other oral surfaces such as the cheek, tongue, and teeth

are added to this number, the best estimate of the total species diversity in the oral cavity is approximately 500 species, as previously proposed.

**DENTAL PLAQUE & FORMATION:**

According to **Nolte WA (1973)[108]** dental plaque has been defined as " the non- mineralized microbial accumulation which adheres tenaciously to the surface of the tooth, restorations and prosthetic appliances, shows structural organization with filamentous forms, and is composed of an organic matrix derived from salivary glycoproteins and extracellular microbial products and cannot be removed by rinsing or spraying water".

Approximately 75% of plaque volume is composed of bacterial cells. The other 25% consists of epithelial cells, leukocytes and macrophages in an inter-microbial matrix.

**Armstrong WG et al. (1968)[6]** stated that within minutes after a tooth surface is freshly cleaned, an acquired pellicle, composed primarily of salivary proteins, is adsorbed to the exposed hydroxyapatite crystallites**.**

**Lie T et al. (1975)[73]** stated that the chemical composition of plaque may be affected by the physical and chemical nature of the surfaces and the distribution of surface charges**.** Because of the rate in which acquired pellicle is formed, it is unlikely that bacterial colonization will begin on any surface without prior formation of an organic coating**.**

**ROLE OF PLAQUE IN PERIODONTAL DISEASE:**

**Loe 's (1965)**[80] experimental gingivitis explained the etiological role of plaque, the initiation and progression of periodontal diseases, but over time ideas on how a transition occurs from oral health to disease have changed.

**Listgarten et al. (1988)**[77]stated that the tooth surface is initially colonized by coccoid, gram-positive, facultative microbial species which adheres to hard surfaces through the elaboration of sticky coatings. These colonies first spread laterally to occupy the available tooth surface and continue to grow as columnar microcolonies in a direction more-or-less perpendicular to the tooth surface. Supra-gingivally, this organized bacterial community is gradually displaced by a more filamentous, gram negative microbiota which forms the dominant bacterial morphotype of mature plaque.

**Listgarten, M. A. (1986)**[76]stated that these changes in the quantity as well as the quality of dental plaque is accompanied in most cases by alterations in the gingival tissues adjacent to the plaque layer. These alterations consist of inflammatory changes which result in gingival oedema and erythema. The oedema contributes to deepening of the sulcus (pseudo-pocket) and the formation of an environment suitable for the establishment of an anaerobic microbiota located sub-gingivally. This flora which may require several weeks to appear in an otherwise healthy site, consists predominantly of gram negative, anaerobic species, including many spirochetes and other motile bacteria.

**NON-SPECIFIC PLAQUE HYPOTHESIS:**

**Miller's (1890)** [95]investigation brought forth the Non-specific plaque hypothesis. He has postulated that the amount of plaque determines the pathogenicity without distinguishing between the levels of bacteria's virulence. According to **Theilade (1986)**[144], the disease occurs when the plaque content with its toxins and breakdown products exceeds the capacity of the host response. He also stated that all plaque bacteria contribute to microflora virulence by playing a role in the colonization and evasion of the defense mechanisms.

**Page RC (1976)** [109]stated that however this hypothesis does not suit for the development of periodontitis, whereas it is applicable only for the development of gingivitis since periodontitis is a multifactorial disease. The main drawback of this hypothesis is that it failed to prove why all gingivitis does not progress to periodontitis. **Socransky et al. (1994)**[137]pointed out that this hypothesis failed to explain as to why in some subjects aggressive and advanced forms of periodontitis had little plaque whereas in some subjects with mild periodontitis had increased amount of plaque and also site specificity of the disease is inconsistent with the concept that all plaque is equally pathogenic.

**SPECIFIC PLAQUE HYPOTHESIS:**

**Walter J. Loesche in (1978)**[81] proposed the specific plaque hypothesis, wherein he stated that the quality but not the quantity of plaque

mattered as only some microorganisms in plaque were considered to be pathogenic. As the number of these particular bacteria increased, virulence factors released by them would lead to periodontal disease. For example, in localized aggressive periodontitis, the specific pathogen is Aggregatibacter actinomycetemcomitans. Following the development and maturation of dental plaque, and with increase in probing depth, the oral microbial flora undergoes a transition from gram positive aerobic species to gram-negative anaerobic species.

The specific microbial groups with dental plaque was identified by **Socransky** and **Haffajee**[135] in **(1998)** and six inter-related groups were reported. The early colonizers consist of green, purple and yellow complexes. These complexes help in the colonization of orange and red complexes. The red complex organisms are associated with periodontitis which includes Tannerella forsythia, Porphyromonas gingivalis and Treponema denticola. The main disadvantage of this hypothesis is that it could not explain as to why Porphyromonas gingivalis and Tannerella forsythia, considered the main putative periodontal pathogens, are also mostly present in healthy periodontium.

**ECOLOGICAL PLAQUE HYPOTHESIS:**

During the **1990s, Marsh and coworkers**[88, 89, 90, 91]developed the "ecologic plaque hypothesis" as an attempt to unify the existing theories regarding the role of dental plaque in periodontal disease.

In this theory, he stated that the disease is the result of an imbalance in the total microflora due to ecological stress, resulting in an enrichment of some "oral pathogens" or disease-related microorganisms.

**Berezow AB &Darveau RP (2011)[9]**stated that the ecologic plaque hypothesis is entirely consistent with observations that disease-associated organisms are minor components of the oral microbiota in health; these organisms are kept in check by interspecies competition during microbial homeostasis. Disease is associated with the overgrowth of specific members of the dental plaque biofilm when the local microenvironment changes, but it is not necessarily the same species in each case.

**Marsh (1994)[88]** stated that dental plaque forms naturally on teeth and is of benefit to the host by helping to prevent colonization by exogenous species. The bacterial composition of plaque remains relatively stable despite regular exposure to minor environmental perturbations. This stability (microbial homeostasis) is due in part to a dynamic balance of both synergistic and antagonistic microbial interactions. However, homeostasis can break down, leading to shifts in the balance of the microflora, thereby predisposing sites to disease.

**KEYSTONE PATHOGEN CONCEPT:**

**Stecher, B. et al. (2007)[140]**stated that a keystone microorganism that supports and stabilizes the dysbiotic microbiota associated with a disease state is referred to here as a keystone pathogen. Importantly, the capacity of

keystone pathogens to instigate inflammation even when they are present as quantitatively minor components of the microbiota is in stark contrast to the inflammation induced by dominant pathogens, which become established as the dominant components of the microbiota while simultaneously causing suppression of the commensal organisms.

**Hajishengallis, G. et al. (2012)[45]** stated that accordingly, it was hypothesized that P. gingivalis impairs innate immunity in ways that alter the growth and development of the entire biofilm, triggering a destructive change in the normally homeostatic host–microbiota interplay in the periodontium. In other words, P. gingivalis could be a keystone pathogen of the disease-provoking periodontal microbiota. In a mouse model study, it was shown that very low colonization levels of P. gingivalis induces periodontitis accompanied by significant alterations in the number and community organization of the oral commensal bacteria.

**Frias-Lopez et al. (2012)[41]** stated that the P. gingivalis exerts a keystone effect via host modulation, one cannot rule out the possibility that this pathogen also modulates the commensal oral microbiota through host-independent, direct effects. Meta-transcriptomic analysis of oral microbial communities has shown that the introduction of P. gingivalis into a healthy multispecies biofilm alters the pattern of community gene expression.

The keystone concept is furthermore consistent with P. gingivalis being a quantitatively minor constituent of human periodontitis-associated biofilms (**Moore, W. E. et al 1982[97] ; Kumar, P. S.et al.2006[70] ;**

**Doungudomdacha, S., 2000)**[32], despite its high prevalence and association with progressive bone loss in periodontitis patients (**Chaves, E. S.et al.2000**[17] **; Moore, W. E. etal.1991**[96]) Importantly, specific removal of P. gingivalis from the periodontal biofilm (by means of a C5aR antagonist) reverses the dysbiotic changes (**Hajishengallis, G. et al. 2012)**[45], indicating that dysbiotic diseases could be treated by specific targeting of keystone pathogens.

**POLYMICROBIAL SYNERGY AND DYSBIOSIS MODEL:**

**Hajishengallis et al (2012)**[45]proposed that periodontitis is initiated by synergistic and dysbiotic microbiota as opposed to the traditional view that it is caused by a group of select pathogens. In asynergistic community, different members/specific gene combinations fulfill distinct roles that bring about a disease-provoking microbiota. So, the key requirement is the presence of certain species which has the ability to modulate the host response and tip the balance towards dysbiosis from homeostasis. The keystone pathogens also elevate the virulence of the entire community by modulating the effects of other pathogens to maintain a pro-inflammatory microbiota that elicits a non-resolving and tissue destructive host response. On the basis of the fundamental concepts underlying this model of periodontal pathogenesis, that is polymicrobial synergy and dysbiosis, this was termed as PSD model.

**Jenkinson & Lamont. (2005)**[56]**& Hansen et al. (2007)**[48] stated that it is becoming evident, therefore, that the virulence of periodontal pathogens such as P. gingivalis acquires importance only in the context of a synergistic

microbial community, which is required for the expression of pathogenicity. This model is consistent with the participation of both gram-negative and gram-positive bacteria in periodontal pathogenesis, as long as they can provoke or tolerate inflammation, or provide other useful service to the community. Mixed microbial communities provide opportunities for competitive and co-operative interspecies interactions, and such interactions shape the nature and function of the entire assemblage.

However, for pathogenic potential to be realized, the activities of a keystone species such as P. gingivalis are required. These organisms engage in two-way communication with the community inhabitants, in particular the accessory pathogens, to both disrupt host immune surveillance and elevate the pathogenicity of the entire group. This more specialized dysbiotic role will be restricted to fewer organisms. The identification of keystone and accessory pathogens from the catalog of organisms generated by microbiome projects will present the next major challenge in periodontal disease research and, perhaps, in other inflammatory diseases with a complex poly microbial etiology **(Hajishengallis et al., 2012) [45].** Moreover, an in depth understanding of the periodontal pathogenesis on the basis of PSD model may offer new targets for therapeutic intervention.

## CALCULUS AND ITS COMPOSITION:

**Socransky (2002)[138]& Schroeder (1969)[127]**stated that dental calculus develops when nonmineralized biofilms, extremely rich in oral bacteria,

become mineralized with calcium phosphate mineral salts. These mineralized biofilms form both supragingivally and subgingivally.

**Hazen SP (1995)[50]**stated that the process of mineralization involves metabolic activities of the bacterial colonies and strengthens the attachment of nonmineralized biofilms to the tooth surface. It thus maintains close proximity to the gingival tissues, as dental biofilms always cover the surface of the mineralized deposits.

**Meuhlemann H, Schroeder H. (1964)[99]**showed that the mineralization process of the biofilm appears to be completed in 12 days, but half of the mineralization occurs in the first 2 days.

**Jepsen S (2011) [57]& Schroeder H (1963 & 1969)[126, 127]**stated that following mineralization, the roughened surface of the calculus provides an ideal ground for the deposition of new biofilm. Calcified and previously calcified biofilms consist of four different types of calcium phosphate crystals

- CaH (PO4) 9 2H2O = Brushite
- Ca4H (PO4)3 9 2H2O = Octacalcium phosphate
- Ca5(PO4)3(OH) = Hydroxyapatite
- β-Ca3 (PO4)2 = Whitlockite.

**Friskopp S (1984) [42]&Kani.T (1983)[60]**stated that supragingival calculus is clearly built up in layers and showed high heterogeneity from one layer to another. On an average, the mineral content is 37%, but ranges from

16% to 51%, with some exceptional layers having a maximal density of minerals of up to 80%.

**THEORIES OF CALCULUS FORMATION:**

Several theories of calculus formation were noted in the literature, but four seemed to stand out:

- The precipitation of calcium salts from the saliva to form calculus is due to the loss of carbon dioxide from the saliva.
- The formation of calculus is due to the presence and activity of bacteria.
- The formation of calculus is due to a colloidal type of precipitation.
- The formation of calculus is due to the activity of the pyrophosphatase enzyme.

1) Loss of Co2:

**Burchard (1895)[11, 12]**postulated that the precipitation of calcium salts from a supersaturated saliva was the result of an increased pH caused by the loss of carbon-dioxide from the saliva.

**Hodge & Leung (1950)[52]** attempted to substantiate this theory by experimentally producing an explanation of the possible mechanism involved. They demonstrated in in vitro experiments that a precipitate would form upon the loss of carbon dioxide from a supersaturated saliva; conversely, with a high carbon dioxide tension, no precipitate formed.

2) Bacteria:

**Naeslund (1925)**[100,101,102]offered evidence that Actinomyces and Leptotrichia were the important organisms in calculus. The Leptotrichia usually formed a distinct, more superficial layer over the deeper Actinomyces colonies. He thought that the growth of these organisms produced certain biochemical changes that lead to precipitation of calcium salts from the saliva (supragingival calculus) or the serum or exudate (subgingival calculus). The precipitates could readily be trapped by the bacteria already attached to surfaces of the teeth.

3) Colloidal Precipitation:

**Prinz (1921)**[119] put forth the idea that colloidal substances in the saliva became viscous and formed a matrix for the precipitation of calculus. These colloidal substances condensed around an 'inanimate nucleus', and inorganic calcium and magnesium salts precipitated out at right angles to this surface, making a radiating form. Colloidal particles then filled in around these salts to produce a laminated appearance. He stated that the alkalinity of the saliva was essential for calculus formation but that this alkalinity was due to ammonia produced from protein decomposition and not due to the loss of carbon-dioxide from the saliva.

4) Enzyme:

**Chauncey H & Helman EZ (1954)**[16]stated that analysis of saliva has found that phosphatase is present in the saliva of both people who form

calculus and those who do not. No significant difference in the level of phosphatase was found between the two groups. Both alkaline and acid phosphatase were found in whole saliva but only acid phosphatase in parotid saliva.

**F.J Draus et al. (1968)**[34] stated that the purpose of this study (Salivary enzymes and calculus formation) was to determine if enzymatic activities could be correlated with calculus formation. Subjects were grouped as to their ability or inability to form calculus after a three month "accumulation" period. Saliva samples were collected and the enzymatic activities of nonspecific esterase, acid phosphatase, pyrophosphatase, neuraminidase, protease, and lactic dehydrogenase were determined by routine analysis. Statistical evaluation of the data using the standard t test indicated that esterase activity has a high correlation to calculus formation, pyrophosphatase activity also correlated with calculus formation. Acid phosphatase activity correlated to a lesser degree. No other significant correlations were found. A possible sequence for salivary calcification is presented.

**Pradeep A.R. (2011)**[118]stated that a large amount of calculus may hamper the efficacy of daily oral hygiene and thereby accelerate plaque formation. Salivary concentrations of orthophosphate and pyrophosphate are important in preventing calculus formation. Activity of orthophosphate, pyrophosphate, and pyrophosphatase was studied in whole saliva in calculus-forming groups and plaque-forming groups. The results are conclusive that the

components orthophosphate, pyrophosphate, and pyrophosphatase present in saliva have a very significant role to play in formation and inhibition of calculus. This study reinforces the idea of using pyrophosphate and newer bisphosphonates as potential anti-calculus agents.

**ROLE OF DENTAL CALCULUS IN PERIODONTAL DISEASE:**

**Ainamo (1970)** [2] found a high positive correlation between calculus (both supra- and subgingival and gingivitis) in 154 army recruits between the ages of 19 and 22. He employed the retention index (RI) which discriminates between plaque associated with calculus and plaque associated with caries and noted a positive correlation between the RI and gingivitis. A higher correlation was noted between gingivitis and calculus related plaque than with cariogenic plaque.

**Alexander (1971)**[4] observed the regional distribution of bacterial plaque, supra- and subgingival calculus, and gingival inflammation in 200 dental students and 200 patients visiting a dental clinic. He noticed that the prevalence of gingival inflammation is greatly exhibited in the papillary areas and the buccal margins the lowest which coincides with the greatest prevalence of subgingival calculus on the interproximal surface and the buccal margins the lowest, concluding that the surfaces with calculus exhibited more gingivitis than the surfaces with plaque alone.

**Patters et al. (1982)**[114] assayed the bone resorbing activity and the presence of antigens of P.gingivalis in plaque, calculus, cementum, and dentin

obtained from roots of extracted teeth from patients with severe periodontitis. Significant stimulation of bone resorption was found in the preparations from periodontally involved cementum and in all samples of calculus. The levels of bone resorbing activity were higher. This study provides the strongest evidence to date of the pathogenic potential of subgingival calculus.

**Mandel ID (1986)[85]** stated that supragingival calculus predisposes to the development of periodontal disease by providing a retentive surface for plaque bacteria and impeding attempts to maintain an effective level of plaque control.

**Nichols FC et al. (2001)[106, 107]**stated that calculus does not contribute directly to gingival inflammation, but it provides a fixed nidus for the continued accumulation of bacterial plaque and its retention in close proximity to the gingiva. Periodontal pathogens such as *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis,* and *Treponema denticola* have been found within the structural channels and lacunae of supragingival and subgingival calculus.

**Jepsen S et al. (2011)[57]**stated that the rough calculus surface may not, by itself, induce inflammation in the adjacent periodontal tissues, instead it serves as an ideal substrate for subgingival microbial colonization and also

• acts as a niche which harbors bacterial plaque

• acts as an irritant to the periodontal tissues

• distends the periodontal pocket wall

• inhibits the ingress of polymorphonuclear leukocytes.

**BIOFILM:**

**Hall-Stoodley L et al. (2012)[47]** defined microbial biofilm as 'an aggregate of microbial cells surrounded by a self-produced polymer matrix', and both monospecies and polyspecies biofilms exist. Biofilms may or may not adhere to surfaces, but they are predominantly situated in the tissue or in secretions, and components from the host may be found in biofilms.

**Anthony van Leeuwenhoek (1632–1723)[31]** observed and described bio-films by using his primitive microscope on matter from his own mouth where he saw aggregated microbes in the "scurf of the teeth" and from "particles scraped off his tongue".

**Slots & Gibbons (1978)[133]** reported that the introduction of P. gingivalis into the mouths of human volunteers resulted in the organism locating almost exclusively on preformed, streptococcal rich supragingival plaque.

**PLAQUE BACTERIA AND ITS INTERACTIONS:**

**Diaz PI et al. (2000)[29]**stated that most nutrients for dental plaque bacteria originate from saliva orgingival crevicular fluid, although the host diet provides an occasional but nevertheless important food supply. The transition from gram-positive to gram-negative microorganisms observed in the structural development of dental plaque is paralleled by a physiologic transition in the developing plaque. The early colonizers (e.g., Streptococcus

and Actinomyces spp.) use oxygen and lower the redox potential of the environment, which then favors the growth of anaerobic species.

**Loesche WJ (1968)[82]** stated that many of the gram-positive early colonizers use sugars as an energy source. The bacteria that predominate in mature plaque are anaerobic and asaccharolytic (i.e., they do not break down sugars), and they use amino acids and small peptides as energy sources.

**Lin X et al. (2006)[75]**stated that nonpathogenic organisms in subgingival dental plaque can modify the behavior of periodontal pathogens. For example, long and short fimbriae of P. gingivalis are required for adhesion and biofilm formation. The expression of long fimbriae is down-regulated in the presence of Streptococcus cristatus, and short fimbriae are downregulated by S. gordonii, S. mitis, or S. sanguinis.

**Van der Hoeven JS. (1976)[148]** stated that the co-existence of bacteria in plaque might be explained in terms of competition for growth limiting substrates, since most natural environments, such as plaque, are nutrient-limited. Indeed, plaque bacteria appear to be carbon and energy limited.

**Rogers AH et al. (1984)[123]**in a study had evaluated, a number of plaque organisms in a glucose limited condition at various growth rates and relevant growth parameters were determined. This was accomplished using the chemostat - a continuous culture device that has a number of features in common with the mouth. Its principal advantage is that microbial growth rates (p) can be controlled by regulating the rate at which the growth-limiting medium enters the culture vessel; that is, the dilution rate. This is of particular

importance in studying the properties and activities of bacteria at growth rates approaching those occurring naturally; indeed, the biochemical activities and cell-surface properties of organisms such as Streptococcusmutans vary according to growth rate and nutrient limitation.

**Hardie & Bowden, (1974)[49]** stated that samples of plaque taken from adjacent areas of the same tooth can show distinct differences in their bacterial populations. Coupled with these differences in the flora there are changes in the bacterial composition which are dictated by the prevailing physiological conditions in specific areas.

**BIOFILM FORMATION:**

**Ximenez-Fyvie LA et al (2000)[153]** in his study, compared the microbial composition of supragingival and subgingival plaque in 22 healthy and periodontitis subjects. A total of 2358 plaque samples were collected from the mesial aspect of all the teeth excluding 3rd molars in each subject. Samples were examined for the presence of 40 bacterial taxa using whole genomic DNA probes and checkerboard DNA-DNA hybridization. Results showed that, Porphyromonas gingivalis, Bacteroides forsythus and Treponema denticola were detected in supragingival plaque samples of both healthy and periodontitis subjects. Actinomyces species was the dominant taxa in both supra- and subgingival plaque obtained from healthy and periodontitis subjects. Increased proportions of P. gingivalis, B. forsythus, and species of Prevotella, Fusobacterium, Campylobacter and Treponema were detected

subgingivally in the periodontitis subjects. P. gingivalis, B. forsythus and T. denticola were significantly more prevalent in both supra and subgingival plaque samples from periodontitis subjects. He concluded that the data obtained from the supra and subgingival plaque samples from the periodontally healthy subjects indicated that while there was a shift in the microbiota during disease, the shift was not so radical that ‘‘re-adjustment’’ back to health could not be reasonably accomplished in the majority of periodontitis subjects. Clearly, decreasing the proportion of ‘‘red’’ and ‘‘orange’’ complex species and decreasing the numbers of organisms in the subgingival space should be desired end points of infection control in periodontitis subjects.

**Quirynen M (2005)[120]**, in his study, stated that the treatment of periodontitis involves the reduction of perio-pathogens and that, after therapy, beneficial and pathogenic species recolonize the subgingival area. His prospective, split-mouth study followed the early colonization of 'pristine' pockets which was created during implant surgery (16 partially edentulous patients), to record the time needed before a complex subgingival flora could be established with the supragingival area as the single source. Four subgingival plaque samples were taken from shallow and medium pockets around implants (test), and neighboring teeth 1, 2, and 4 weeks after abutment connection. Checkerboard DNA-DNA hybridization and culture data revealed a complex microbiota in the pristine pockets within a week, with a minimal increase in counts up to 4 weeks. Analysis of these data demonstrated that,

even with the supragingival environment as the single source for colonizing bacteria, a complex subgingival microbiota can develop within one week.

**Yamada M et. al. (2005)[154]** stated that biofilm formation is an important step in the etiology of periodontal diseases. In his study, in vitro biofilm formation by Treponema denticola and Porphyromonas gingivalis 381 displayed synergistic effects. Confocal microscopy demonstrated that P. gingivalis attaches to the substratum first as a primary colonizer followed by coaggregation with T. denticola to form a mixed biofilm. The T. denticola flagella mutant as well as the cytoplasmic filament mutant were shown to be essential for biofilm formation as well as coaggregation with P. gingivalis. The major fimbriae and Arg-gingipain B of P. gingivalis also play important roles in biofilm formation with T. denticola.

**Capestany CA et al. [14]**stated that Clp proteases and chaperones are ubiquitous among prokaryotes and eukaryotes, and in many pathogenic bacteria, the Clp stress response system is also involved in regulation of virulence properties. In his study, the roles of ClpB, ClpC, and ClpXP in stress resistance, homotypic and heterotypic biofilm formation, and intracellular invasion of the oral opportunistic pathogen, *Porphyromonas gingivalis* were investigated. Absence of ClpC and ClpXP, but not ClpB, resulted in diminished tolerance to high temperature. Response to oxidative stress was not affected by the loss of any of the Clp proteins. The *clpC* and *clpXP*mutants demonstrated elevated monospecies biofilm formation, and the absence of ClpXP also enhanced heterotypic *P. gingivalis-Streptococcus gordonii* biofilm

formation. All *clp* mutants adhered to gingival epithelial cells to the same level as the wild type; however, ClpC and ClpXP were found to be necessary for entry into host epithelial cells. ClpB did not play a role in entry but was required for intracellular replication and survival. ClpXP negatively regulated the surface exposure of the minor fimbrial protein subunit of *P. gingivalis*, which stimulates biofilm formation but interferes with epithelial cell entry. Collectively, these results show that the Clp protease complex and chaperones control several processes that are important for the colonization and survival of *P. gingivalis* in the oral cavity.

**Nakao et al. (2006)** [104]stated that *Porphyromonas gingivalis* is a crucial component of complex plaque biofilms that form in the oral cavity, resulting in the progression of periodontal disease. To elucidate the mechanism of periodontal biofilm formation, the involvement of several genes related to the synthesis of polysaccharides in *P. gingivalis* were analyzed. He concluded that the *galE* gene plays a pivotal role in the modification of LPS O antigen and biofilm formation in *P. gingivalis* and considered that his findings of a relationship between the function of the *P. gingivalisgalE*gene and virulence phenotypes such as biofilm formation may provide clues for understanding the mechanism of pathogenicity in periodontal disease.

**VIRULENCE OF BACTERIA:**

**Curtis MA (2005)**[23]stated that the virulence of a microbe represents a combination of complex factors including the agent's transmissibility and the

severity of the disease associated with infection and is also significantly influenced by the susceptibility of the colonized host. Virulence factors may be defined as those products of the organism which are required to complete the various stages of the life cycle leading to pathology in the host.

**Falkow (1988)**[39]stated that the virulence determinants of a pathogen can simply be defined as those gene products which facilitate colonization, growth and survival within the diseased host organism and spread to a new host.

**TABLE NO: 1**

| BACTERIA | DESCRIPTION | VIRULENCE FACTORS |
|---|---|---|
| 1) Aggregatibacter actinomycetemcomitans[134, 137] | Gram positive, non-motile, Associated with aggressive periodontitis | - Leukotoxin<br>- Cytolethal Distending Toxin (CDT)<br>- Fc binding protein<br>- Collagenases<br>- Lipopolysaccharides<br>- Proteases<br>- cagE-homologue<br>- OMP-1<br>- OMP-100<br>- GroEL (Hsp60)<br>- 65 KDa protein<br>- Surface proteins (SAM :14-79 kDa) |

| | | |
|---|---|---|
| 2) Porphyromonas Gingivalis[103,146,75] | Gram negative, non-motile, anaerobic pathogenic bacteria | - Gingipain<br>- Capsular polysaccharide<br>- Hemolysins<br>- Volatile sulfur compounds<br>- Ammonia<br>- Indole<br>- Fimbriae |
| 3) Tannerella Forsythia[129] | Gram negative anaerobic bacteria | - Lipoproteins (BfLP)<br>- Leucine rich repeat protein (BspA) |
| 4) Treponema Denticola[27] | Gram negative anaerobic motile bacteria | - Lipoplysaccharides<br>- Proteases<br>- Dentilisin<br>- Major Surface Protein (MSP)<br>- Leucine rich repeat proteins |

**Liu H et al. (2007)** [79]stated that adhesins can be subdivided into two major classes: fimbrial adhesins, including fimbriae, pili, curli and type IV pili, and nonfimbrial adhesins, such as autotransporter, outer membrane and secreted adhesins, and those associated with biofilm formation. Many bacteria express an entire set of various adhesins, often belonging to different subclasses, on their surfaces, which might be an adaptation to different phases of pathogenesis.

**Darveau RP et al. (1997)** [25]stated that fimbriae are thought to play important roles in the expression of virulence by various periodontal microbes, including P. gingivalis and Aggregatibacter actinomycetemcomitans. Fimbriae– host interactions precede the infection process induced by these pathogens, and high titers of antibodies to fimbriae are often found in the sera of patients following infection. Thus, bacterial fimbriae have attracted researchers seeking potential vaccines against periodontal pathogens.

**Henderson et al**. (**1996)**[51] introduced the modulin concept to estimate local immunocompromising factors as etiologic agents of periodontal diseases. Although their estimation of modulin is limited to innate and acquired immuno-response, it is quite helpful for identifying subgingival virulence factors, such as tissue damaging enzymes, exotoxin, and endotoxin.

**Kato T et al. (2007)**[62] stated that Porphyromonas gingivalis is a periodontal pathogen whose fimbriae are classified into six genotypes based on the diversity of the fimA genes encoding each fimbria subunit. It was suggested that P. gingivalis strains with type II fimbriae were more virulent than type I strains. For the present study, he generated the mutants in which fimA was substituted with different genotypes to study virulence of type II fimbriae. Using plasmid vectors, fimA of ATCC33277 (type I strain) was substituted with type II fimA, and that of OMZ314 (type II strain) with type I fimA. The substitution of type I fimA with type II enhanced bacterial adhesion/invasion to epithelial cells, whereas substitution with type I fimA resulted in diminished efficiency. Following bacterial invasion, type II clones

swiftly degraded cellular paxillin and focal adhesion kinase, and inhibited cellular migration, whereas type I clones and DeltafimA mutants did not. BIAcore analysis demonstrated that type II fimbriae possess greater adhesive abilities for their receptor alpha5beta1-integrin than those of type I. In a mouse abscess model, the type II clones significantly induced serum IL-1beta and IL-6, as well as other infectious symptoms. These results suggest that type II fimbriae are a critical determinant of P. gingivalis virulence.

**Lamont RJ (1998)[71]** stated that the pathogenicity of periodontal diseases is undoubtedly complex, with multiple bacteria with various virulence factors interacting with a variety of host cells and immune reactions. However, strong evidence exists in this context to support the role of intercellular and intracellular invasion in the initiation and progression of disease. It is not difficult to imagine that the presence of organisms in the gingiva such as highly proteolytic P.gingivalis or leukotoxic A.actinomycetemcomitans would not be beneficial to the host's health status. The closer proximity to the targets would enable destructive bacterial products such as proteases, leukotoxins, lipopolysaccharides, etc. to cause greater devastation to the structural integrity of the periodontal tissue.

**Tanner et al. (1986)[142]**stated that T. forsythia was first reported and is a nutritionally fastidious anaerobe. While available data on its complement of virulence factors remain to be described, use of enzymatic speciation profiles (i.e. the APIZYME system) demonstrates that this species produces a wide

array of activities, through proteases, that could degrade extracellular matrix, and biomolecules that could undermine host response factors. Thus, this bacterium has the capacity to contribute multiple properties to the characteristics of the microenvironmental milieu of the biofilm.

**MICROBIAL COLONIZATION:**

**Kolenbrander PE (2006)[66]** stated that the primary colonizing bacteria which adhere to the tooth surface provide new receptors for attachment by other bacteria as part of a process known as co-adhesion. Together with the growth of adherent microorganisms, co-adhesion leads to the development of microcolonies and eventually to a mature biofilm.

Coaggregation is a direct interaction; it is distinct from agglutination, which occurs when cells are stuck together by molecules in solution. At least 18 genera from the oral cavity have shown some form of coaggregation.

**Doyle RJ et al**. (**1990)[33]** stated that the initial stages of coaggregation or co-adhesion are essentially the same as the first steps involved in bacterial binding to surfaces: bacterial cells come into contact through passive or active transport and bind weakly through nonspecific hydrophobic, electrostatic, and van der Waals forces.

**Kolenbrander PE et al. (1993)[65]** stated that different species or even different strains of a single species have distinct sets of coaggregation partners. Fusobacteria co-aggregate with all other human oral bacteria,

whereas Veillonella spp., Capnocytophaga spp. and Prevotella spp. bind with streptococci and/or actinomyces.

**Kaplan CW et al. (2009)[61]**stated that well-characterized interactions of secondary colonizers with early colonizers include the coaggregation of F. nucleatum with S. sanguinis, Prevotella loescheiiwith A. oris, and Capnocytophagaochraceawith A. oris. Secondary colonizers such as P. intermedia, P. loescheii, Capnocytophaga spp., F. nucleatum, and P. gingivalis do not initially colonize clean tooth surfaces but rather adhere to bacteria that are already in the plaque mass.

**Cisar JOet al. (1993)[19]**stated that the idea of that coaggregation is important during the formation of oral biofilms opens new perspectives, especially for the use of probiotics. Special examples of coaggregations are the corn cob formation, in which streptococci adhere to filaments of Corynebacterium matruchotiior Actinomyces spp., and the test tube brush formation, composed of filamentous bacteria to which gram-negative rods adhere.

**Socransky SS et al. (1998)[135]** stated that the microorganisms primarily considered secondary colonizers fell into the green, orange, and red complexes. The green complex includes Eikenella corrodens, A.actinomycetemcomitans serotype a, and Capnocytophaga spp. The orange complex includes Fusobacterium, Prevotella, and Campylobacter spp.The green and orange complexes include species recognized as pathogens in periodontal and nonperiodontal infections. The red complex consists of P.

gingivalis,T. forsythia, and T. denticola. This complex is of particular interest because it is associated with bleeding on probing.

**MICROBIAL INDENTIFICATION AND INVESTIGATION:**

Microbial cultures are used to determine the type of organism, its abundance in the sample being tested, or both. It is one of the primary diagnostic methods of microbiology and used as a tool to determine the cause of infectious disease by letting the agent multiply in a predetermined medium.

**Walker CB et al. (1979)[150]** stated that a selective medium, CVE (Crystal -Violet Erythromycin) agar, was developed for the isolation of Fusobacterium nucleatum from subgingival plaque of periodontally diseased patients. The medium contained 1.0% Trypticase (BBL Microbiology Systems), 0.5% yeast extract, 0.5% NaCl, 0.2% glucose, 0.02% L-tryptophan, 1.5% agar, and 5% defibrinated whole sheep blood. Erythromycin and crystal violet were added as the selective inhibitory agents at concentrations of 4 and 5 micrograms/ml, respectively. The medium permitted almost total recovery of F. nucleatum when compared with a non-selective medium and suppressed the recovery of most remaining species by nonselective medium by 6 to 8 orders of magnitude. Microorganisms suppressed to a lesser degree included Selenomonassputigena, Actinobacillusactinomycetemcomitans, Eikenella corrodens, and some strains of Peptostreptococcus. The distinct colonial morphology of F. nucleatum on CVE agar made differentiation relatively easy

when contaminants were present. With this medium, F. nucleatum was enumerated from 278 subgingival plaque samples and accounted for less than 1% to greater than 25% of the cultivatable microbiota.

**Tempro PJ et al. (1986)[143]** developed a medium for the selective recovery of Haemophilus aphrophilus. The medium, designated TSBVF, was composed of 4% tryptic soy agar, 10% heat-inactivated horse serum, 75 micrograms of bacitracin per ml, 5 micrograms of vancomycin per ml, and 50 micrograms of sodium fluoride per ml. TSBVF yielded a threefold higher recovery of oral H. aphrophilus than did chocolate agar with 75 micrograms of bacitracin per ml, which is a medium routinely used to diagnose human Haemophilus infections. H. aphrophilus and the few contaminating organisms on TSBVF were readily distinguished on the basis of colony morphology. The H. aphrophilus isolates exhibited variable fermentation of raffinose and dextrin but otherwise were biochemically similar. In a clinical study, H. aphrophilus was frequently recovered from supragingival plaque and saliva and occasionally from buccal mucosa and the tonsils. It was also isolated from 29 of 56 subgingival sites in 11 of 14 subjects. The proportion of the subgingival microflora averaged 0.13% for healthy periodontal sites, 0.05% for adult periodontitis lesions, and 0.03% for localized juvenile periodontitis lesions. It was concluded that H. aphrophilus is an indigenous bacterium of the human oral cavity. It occurs in low proportions in subgingival plaque and plays no apparent role in advanced periodontal disease in humans.

**Holm. A et. al (1987)** [53] stated that by modifying the previously described media tryptic soy-serum-bacitracin-vancomycin (TSBV) agar and tryptic soy-serum-bacitracin-vancomycin-fluoride (TSBVF) agar, two improved selective culture media were developed for isolation and enumeration of Aggregatibacter actinomycetemcomitans (A medium) and Haemophilus aphrophilus (H medium) in oral specimens. Both mediums were supplemented with fusidic acid and spiramycin, and carbenicillin was also added to A medium. The growth yields of pure cultures of A. actinomycetemcomitans on A medium and of H. aphrophilus on H medium were comparable with those on the reference media. Compared with blood agar, the selective media inhibited these species about 10-fold or less. In addition, A and H media suppressed the growth of pure cultures of Capnocytophaga spp. and Neisseria spp., commonly found as contaminants on TSBV and TSBVF, 10 times or more compared with that on blood agar. In samples from diseased periodontal pockets, the recoveries of A. actinomycetemcomitans on A medium and H. aphrophilus on H medium equaled those on TSBV and TSBVF, respectively. In about 50% of the cultures on the reference media, contaminating bacteria were detected at levels higher than 10 CFU/ml of sample. The corresponding value for both A and H media was about 2%.

**Alsina M et al. (2001)**[5] stated that Aggregatibacter actinomycetemcomitans is considered to be one of the major oral putative pathogens, especially in cases of juvenile periodontitis. This microorganism

requires nutritionally complex media for growth, and therefore the media for its primary isolation usually include blood agar or serum in their base. This study presented a new medium, Dentaid-1, with improved detection of A. actinomycetemcomitans in periodontal samples. In its composition, blood and serum have been omitted, hence reducing its cost and making it a more restrictive medium against the growth of other microorganisms with high nutritional requirements. The growth yields of pure cultures of the bacteria on Dentaid-1 were comparable to those on nonselective blood agar. Moreover, clinical efficacy was evaluated in subgingival samples from 77 subjects with adult periodontitis. Dentaid-1 detected A. actinomycetemcomitans in 24 subjects, while a previously described tryptic soy-serum-bacitracin-vancomycin agar detected the microorganism in only 19 subjects (79.1%). Dentaid-1 is a low-cost, non-inhibitory formula for the improved diagnosis and monitoring of patients sub-gingivally infected by this important oral putative pathogen.

**CULTURE DEPENDENT APPROACHES:**

**Paster BJ & Dewhirst FE (2009)[113]** stated that the culture-dependent approaches to identify microbes involve growing the microorganisms on defined media, followed by identification based on phenotypic and biochemical criteria, differential staining methods, metabolic end-product analysis and cell membrane composition.

**Genco RJ. et al**. (**2000)**[43] stated that culture-dependent approaches are extremely useful for determining the antibiotic susceptibility of oral microbes and for assessing the pathogenicity of individual species.

**Rappe´ MS, Giovannoni SJ (2003)**[121] stated that currently, the culture-dependent approach which may involve extracting nucleic acid from a single colony, cloning the sequence into a plasmid vector, sequencing the ribosomal RNA genes and identifying the sequence using a ribosomal RNA database.

**CULTURE INDEPENDENT APPROACHES:**

**Counting clones and sequencing:**

**Aas JA et al. (2005)**[1] stated that phylogenetic approaches using 16S ribosomal RNA gene clone libraries have been applied to investigate the diversity of culturable and nonculturable species in the human oral cavity.

**Forns X et al. (1997)** [40] stated that the method is labor- and time-intensive, nonquantitative and there is a significant cloning bias inherent with the method.

**Checkerboard hybridization:**

**Darout IA et al. (2002)**[24]stated that the checkerboard hybridization method which involves extracting DNA from oral samples and hybridizing the sample against labeled probes representing whole genomes or 16S ribosomal RNA genes of known microbes. The reason it is called checkerboard is that

the genome or ribosomal RNA probes are hybridized at right-angles to the DNA of multiple oral samples, and processed images of the hybridizations look like a checkerboard.

The checkerboard DNA– DNA hybridization technique has been widely used to comprehensively examine the types and numbers of bacteria in supragingival and subgingival plaque and saliva in healthy subjects and in patients with periodontitis.

**Socransky SS et al. (1998)[135]** analyzed 185 subjects, representing about 13,000 plaque samples, using whole genomic DNA probes to 40 culturable bacterial species in checkerboard hybridization assays to define bacterial complexes, rather than individual species, that were involved in oral health and periodontal disease. In 2005 he stated that this technique is rapid, sensitive and relatively inexpensive, nonspecific target binding is still a major shortcoming.

**Quantitative PCR:**

**Shelburne CE et al. (2000)[130]** stated that quantitative reverse-transcription (RT) PCR allows the detection and quantification of genes / bacteria in microbiological samples. Quantitative RT-PCR using the TaqMan system was first used to quantify Tannerella forsythia in subgingival plaque samples and to measure the density of P. gingivalis and the total number of bacterial cells in plaque samples.

**Asai Y et al**. (**2002)**[7] stated that the quantitative RT-PCR technique is a highly selective method that may be considered as a 'Gold Standard' when quantitative analysis of specific bacteria is needed. However, this method is only able to detect and to quantify known species, and thus is inadequate for the analysis of complex microbial communities containing unidentified species.

**Pyrosequencing:**

**Pozhitkov A et al. (2005)**[116] stated that the core technology of pyrosequencing relies upon a sequence of enzyme-triggered reactions, which ultimately results in the production of a luminescence signal. The enzyme cascade is activated by the generation of a pyrophosphate, which is released after successful incorporation of a nucleotide into the DNA sequence. The pyrophosphate is converted into ATP by an ATP sulfurylase and subsequently used by the luciferase to convert luciferin to oxyluciferin, yielding a luminescence signal.

To perform pyrosequencing, a mix of a nucleic acid template and primers is prepared and deoxyribonucleotide triphosphates are added in a known order (e.g. A, T, G, C, A…) to a reaction vessel. Because the order in which the deoxyribonucleotide triphosphates are added is known and a charge-coupled device camera records the intensity of the luminescence bursts, the template sequence and its quantity can be reconstructed.

**HIGH THROUGH PUT APPROACHES – MICROARRAYS:**

**Colombo AP et al. (2009)[22]** stated that oligonucleotide microarrays have been widely used to identify microorganisms and determine the expression of genes. Microarrays contain oligonucleotide probes that target RNA and DNA genes in biological samples. All microarray platforms share the common attribute that a sensor detects a signal from target sequences which hybridize to immobilized oligonucleotide probes. Both high- and low-density DNA microarrays have been used to identify microbes in the oral cavity.

**Huyghe et al**. (**2008)[55]** was able to classify PCR amplified ribosomal RNA genes to various nodes on a phylogenetic tree using high-density microarrays. Each node on the tree was represented by a hierarchically nested probe. Therefore, a target hybridized to a microarray could be classified to a specific taxonomic level (e.g. phyla, family, genera and species). A potential advantage of this approach is that probe hybridization signals of an 'uncharacterized' ribosomal RNA gene could be fit onto the tree.

**Huyghe et al. (2008)[55]** showed that the hierarchically nested probes approach could detect a single bacterial species spiked into a complex oral sample, the utility of the approach to detect microorganisms in a complex sample has yet to be fully demonstrated or properly evaluated.

**NEXT GENERATION SEQUENCING:**

The massively parallel sequencing technology known as next-generation sequencing (NGS) has revolutionized the biological sciences. With its ultra-high throughput, scalability, and speed, NGS enables researchers to perform a wide variety of applications and study biological systems at a level never before possible.

**First-generation next-generation sequencing:**

Two consistent themes of first-generation systems are: the ligation of oligonucleotide adaptors to DNA fragments and the immobilization of the fragments to a solid surface, such as a bead. The purpose of the adaptors is two-fold: to anchor the fragments to a solid surface; and to serve as primers for amplification and or sequencing.

**Metzker ML. (2010)[94]** stated that the Illumina / Solexa GA system is very different from 454 sequencing. Specifically, in the Solexa system, the targets are amplified on a solid surface. After amplification, only one of the strands is sequenced with all four deoxyribonucleotide triphosphates present during sequencing. Each deoxyribonucleotide triphosphate has a unique fluorophore. Reversible terminator nucleotides also called 'cyclic reversible termination' are used to prevent the insertion of multiple nucleotide bases in the same cycle.

**Second Generation Next Generation Sequencing:**

**Voelkerding KV et al. (2009)[149]** stated that Second-generation next-generation sequencing involves sequencing single molecules in real time, does not involve DNA amplification and thus eliminates error rates associated with PCR and intensity biases.

The Pacific Biosystem system allows detection of single molecules in real time using phospho-linked nucleotides for incorporation with each nucleotide having a different fluorescent label. The advantage of these deoxyribonucleotide triphosphates is that the phospholinked fluorophores are released from the base when it is incorporated in the DNA sequence by the polymerase.

**Eid Jet al. (2009)[37]** stated that the strength of the Pacific Biosystem system is that it allows very long reads (>1,000 nucleotides). Their weakness lies in their highest error rate (ca. 17%) (31) of all next-generation sequencing systems. For example, of the 27 errors in 158 bases, 12 were deletions, eight were insertions and seven were mismatches.

**Third Generation Next Generation Sequencing:**

**Branton D et al. (2008)[10]** stated that the third-generation next-generation sequencing systems were not commercially available. However, the development of these systems is being revealed in the scientific literature. These systems seem to be label-free, do not require the analytes to be immobilized on a surface, do not depend on DNA polymerase, allow single

molecule analysis with a high signal-to-noise ratio and have the potential to sequence DNA strands at high speed and low cost.

The idea behind nanopore technology is that a strand of nucleic acid can be electrophoretically driven through a nanopore, with each nucleotide base modulating the ionic current as it passes through the nanopore.

**Branton et al**. **(2008)**[10]concludes that future nanopore systems will probably be based on nanopores that are hybrids between solid-state (e.g. NAB sys) and alpha-hemolysins (e.g. Oxford Nanopore Technology), and that improvements in electronic sensing using tunneling probes or capacitors will probably improve the detection of single bases through nanopores. Ultimately, nanopore technology promises to result in faster and inexpensive DNA sequencing.

## NEXT GENERATION SEQUENCING IN PERIODONTAL DISEASES:

### GINGIVITIS

**Shi Huang et al. (2011)**[131] stated that the oral microbiome from gingivitis and healthy subjects could be distinguished based on the distinct community structures of plaque microbiomes, but not the salivary microbiomes. Eight predominant taxa were found associated with gingivitis: TM7, Leptotrichia, Selenomonas, Streptococcus, Veillonella, Prevotella, Lautropia, and Haemophilus. Furthermore, 98 species-level OTUs were identified to be gingivitis-associated, which provided microbial features of

gingivitis at a species resolution. For the two selected genera Streptococcus and Fusobacterium, Real-Time PCR based quantification of relative bacterial abundance validated the pyrosequencing-based results. This study suggests that oral samples from the patient population of gingivitis can be characterized via plaque microbiome by pyrosequencing the 16 S rDNA genes. Further studies that characterize serial samples from subjects (longitudinal study design) with a larger population size may provide insight into the temporal and ecological features of oral microbial communities in clinically-defined states of gingivitis.

**James O. Kistler et al. (2013)[64]** stated that current knowledge of the microbial composition of dental plaque in early gingivitis is based largely on microscopy and cultural methods, which do not provide a comprehensive description of oral microbial communities. This study used 454-pyrosequencing of the V1–V3 region of 16S rRNA genes (approximately 500 bp), and bacterial culture, to characterize the composition of plaque during the transition from periodontal health to gingivitis. Plaque samples were analyzed at baseline, and after one and two weeks. In addition, plaque samples from 20 chronic periodontitis patients were analyzed for cross-sectional comparison to the experimental gingivitis cohort. Principal coordinates analysis (PCoA) plots revealed significant shifts in the bacterial community structure of plaque as gingivitis was induced, and community diversity increased significantly after two weeks. Changes in the relative abundance of OTUs during the transition from health to gingivitis were correlated to bleeding on probing (BoP) scores

and resulted in the identification of new health- and gingivitis-associated taxa. Taxa associated with gingivitis included Fusobacterium nucleatum subsp. polymorphum, Lachnospiraceae [G-2] sp. HOT100, Lautropia sp. HOTA94, and Prevotella oulorum, whilst Rothiadentocariosa was associated with periodontal health.

PERIODONTITIS:

**Kumar PS et al. (2005)[69]** stated that most studies of the bacterial etiology of periodontitis have used either culture-based or targeted DNA approaches, and so it is likely that pathogens remain undiscovered. The purpose of this study was to use culture-independent, quantitative analysis of biofilms associated with chronic periodontitis and periodontal health to identify pathogens and beneficial species. Samples from subjects with periodontitis and controls were analyzed using ribosomal 16S cloning and sequencing. Several genera, many of them uncultivated, were associated with periodontitis, the most numerous of which were gram positive, including Peptostreptococcus and Filifactor. The genera Megasphaera and Desulfobulbus were elevated in periodontitis, and the levels of several species or phylotypes of Campylobacter, Selenomonas, Deferribacteres, Dialister, Catonella, Tannerella, Streptococcus, Atopobium, Eubacterium, and Treponema were elevated in disease. Streptococcus and Veillonella spp. were found in high numbers in all samples and accounted for a significantly greater fraction of the microbial community in healthy subjects than in those with periodontitis. The microbial profile of periodontal health also included the

less-abundant genera Campylobacter, Abiotrophia, Gemella, Capnocytophaga, and Neisseria. These newly identified candidates outnumbered Porphyromonas gingivalis and other species previously implicated as periodontopathogens, and it is not clear if newly identified may play a more important role in pathogenesis. Finally, more differences were found in the bacterial profile between subjects with periodontitis and healthy subjects than between deep and shallow sites within the same subject. This suggests that chronic periodontitis is the result of a global perturbation of the oral bacterial ecology rather than a disease-site specific microbial shift.

**Ann L Griffen et al. (2012)[3]** stated that, recent advances in DNA sequencing and bioinformatics technologies have made possible two orders of magnitude higher resolution of bacterial community composition. This study was carried out using 454 pyrosequencing of subgingival samples from a group of individuals with chronic periodontitis and matched periodontally healthy controls, showed fundamental and clear differences in community composition that were not evident using less-powerful approaches. This work confirms previous findings that certain species are more common in disease, but provides a much broader picture of overall community differences and a much deeper look at community complexity, thereby expanding knowledge of putative pathogenic species.

**Payungporn et al (2017)[115]** performed bacterial classifications based on next-generation sequencing (NGS) of 16S rDNA to identify potential bacterial species associated with periodontal disease in Thai patients. Dental

plaque samples were collected from five healthy controls and five patients with chronic periodontitis. Total DNA was extracted and then amplified by specific primers within a V3/V4 region of the 16S rDNA gene. The purified DNA from samples within the same group were pooled together and used to construct DNA libraries with different indexes. High-throughput sequencing with paired-end was carried out on a MiSeq platform. Pass-filter sequencing reads were used for bacterial classification. The comparative analysis of healthy controls and patients with chronic periodontitis revealed that *Porphyromonas gingivalis* and *Prevotella intermedia* were significantly associated with periodontal disease. Other bacteria such as *Treponema denticola, T.medium, Tannerella forsythia, P. endodontalis*and *Filifactoralocis*might be potentially associated with the periodontal disease in Thai patients. They concluded that, several potential bacteria might be associated with periodontal disease in Thai patients and that the obtained data from this study would be useful for understanding the bacterial communities responsible for periodontal disease which might be applied for more specific bacteria-targeted antimicrobial therapy of the disease.

PERI-IMPLANTITIS:

**Tatsuro Koyyangi et al. (2010)[68]** stated that Peri-implantitis (PI) is an inflammatory disease which leads to the destruction of soft and hard tissues around osseo-integrated implants. The purpose of this study was to identify the microbiota in subjects who have PI, clinically healthy implants, and

periodontitis-affected teeth using 16S rRNA gene clone library analysis to clarify the microbial differences. Subgingival plaque samples were taken from the deepest pockets using sterile paper points. Prevalence and identity of bacteria were analyzed using a 16S rRNA gene clone library technique. Results showed that a total number of 112 different species were identified from 335 clones sequenced. Among the 112 species, 51 (46%) were uncultivated phylotypes, of which 22 were novel phylotypes. The numbers of bacterial species identified at the sites of PI, periodontitis, and periodontally healthy implants were 77, 57, and 12, respectively. Microbiota in PI mainly included Gram-negative species and the composition was more diverse when compared to that of the healthy implant and periodontitis. The phyla Chloroflexi, Tenericutes, and Synergistetes were only detected at PI sites. Low levels of periodontopathic bacteria, such as Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans, were seen in peri-implant lesions. He concluded that the biofilm in PI showed a more complex microbiota when compared to periodontitis and periodontally healthy teeth, and it was mainly composed of Gram-negative anaerobic bacteria. Common periodontopathic bacteria showed low prevalence, and several bacteria were identified as candidate pathogens in PI.

**Sanz-Martin et al. (2017)**[125] stated that the peri-implantitis microbiome is commensal-depleted and pathogen-enriched, harboring traditional and new pathogens. The core peri-implant microbiome harbors taxa from genera often associated with periodontal inflammation. The use of next

generation sequencing to explore the microbiome of healthy and diseased peri-implant sites has allowed to expand the breadth of knowledge of the etiology of this disease. QIIME and HOMINGS were used to analyze Illumina MiSeq-generated reads and demonstrated that QIIME and HOMINGS agreed in large part at the genus level. They showed that those pipelines should be complementary: to a certain extent QIIME provided greater breadth of classification whereas HOMINGS provided increased precision. Hence, it was stated that these two accepted and complementary techniques will shed more light on a difficult classification.

# *Materials & Methods*

# MATERIALS AND METHODS

**Study population:**

A sample size of 8 patients was chosen for the study due to factors viz high cost and complexity of the technique involved as reported by **Zheng et al[160], Dzink et al[35]**.

Eight individuals, who came to the Outpatient Department of Periodontics, Ragas Dental College were recruited in the present study, of which 4 were periodontally healthy (control group) and 4 had plaque covering supragingival calculus (test group).

Control group consisted of subjects who had a clinically non-inflamed, healthy gingiva with bleeding on probing (BOP)<10%, probing pocket depth (PPD) less than ≤3mm and no clinical attachment loss (CAL).

Test group comprised of subjects with plaque covering supragingival calculus with bleeding on probing (BOP)> 20%, PPD≤ 3mm and no CAL.

The study protocol was explained, and written informed consent was obtained from each individual. Past dental and medical histories were obtained and then the clinical periodontal examination and plaque sampling were carried out.

**Inclusion criteria**

- Subjectswho do not have any known systemic illness
- Subjects with periodontal health and those with plaque covering supragingival calculus as described earlier were included in the study.

**Exclusion criteria**

- Patients with systemic disorders, like diabetes mellitus,immunological disorders or HIV that may be a contributing factor forperiodontal disease.
- Patients who were under medication of drugs that would alter the microbial characteristics viz. immunosuppressant drugs or steroids.
- Smokers or tobacco users.
- Patient with previous history of periodontal treatment in the past 6months.
- Patient who had been prescribed antimicrobial therapy for past 6months.

**Plaque sampling**

All examinations were done by a single, calibrated examiner. For the test group, plaque was collected from sites that had supragingival calculus and that had shown bleeding on probing. Plaque covering supragingival calculus was gently removed using sterile Gracey's curette. The tip of the curette was

then inserted in an Eppendorf tube which contained ionized molecular water and shaken until the plaque was removed from the curette. For the healthy plaque samples, the sites that didnot show anysign of inflammation and bleeding on probing were chosen. The same process was then followed for sampling from these sites.

The samples thus obtained were frozen and stored at the temperature of -20°C until the sample collection was completed. The samples were collected in 2 days' time and sent for processing so as to avoid any degradation.

**DNA extraction, 16S rRNA amplification, library construction and Sequencing**

The genomic DNA was isolated from 4 plaque samples each of periodontally healthy and those from sites covering the supragingival calculus with the Fast DNA kit and the FastPrep24-5G instrument according to manufacturer's protocol (MP Biomedicals,Santa Ana, CA).

The extracted DNA was first purified along silica-based spin filters (FastDNA kit) and DNA was amplified using the 16S V3 (341F) forward and V4 (805R) reverse primer pairs with additional Illumina adapter overhang nucleotide sequences.

Amplicon synthesis was executed by using thermocycling along 8.5 µl of genomic DNA, 2 µl of amplicon PCR forward primer (2.5 µM), 2 µl of amplicon PCR reverse primer (2.5 µM), and12.5 µl of 2x KAPA HiFiHotStart Ready Mix (KapaBiosystems) at 95 °C primary denaturation for 3 min,

followed by 25 cycles of 95 °C for 30 s, 62.3 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min.

The reactions were cleaned with AgencourtAMPure XP beads (Beckman Coulter Genomics) according to the prescribed methods. The attachment of dual indices and Illumina sequencing adapters were performed by using 5 µl of amplicon PCR product DNA, 5 µl of Nextera XT Index Primer 2 (S5xx), 5 µl of IlluminaNextera XT Index Primer 1 (N7xx), 25 µl of 2x KAPA HiFiHotStart Ready Mix, and 10 µl of PCR-grade water, with thermocycling at 95 °C for 3 min, followed by 8 cycles of 95 °C for 30s, 55 °C for 30s, and 72 °C for 30s, and a final extension at 72 °C for 5 min.

Constructed 16S metagenomic libraries were purified with AgencourtAMPure XP beads and measured with Quant-iTPicoGreen and the KAPA Library Quantification Kit (KAPABIOSYSTEMS). Library quality control was carried out with the Agilent Technologies 2100 Bioanalyzer to confirm the quality and average size distribution.

Samples were denatured and then diluted to a final concentration of 10 pM with a 20 % PhiX (Illumina) control. After that the sequencing was carried out by using IlluminaNextseq 500 System. All 8 samples were multiplexed and sequenced in single lane on the NextSeq by using 2 × 150 bp paired-end sequencing. Data analysis was then done by using 16s metagenomics tool from Base Space Onsite Operational taxonomic units (OTUs) which was assigned to each sequence using HOMD database.

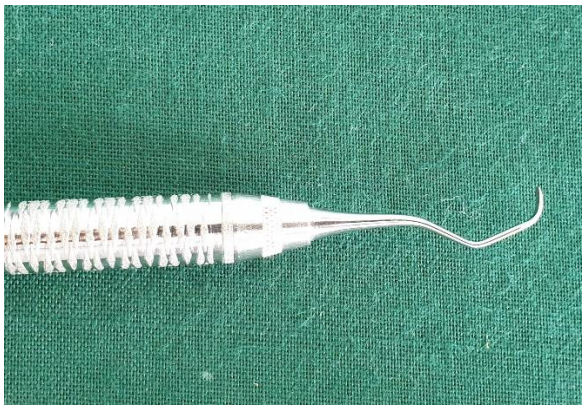
**STATISTICAL ANALYSIS:**

Due to individual variations in the microbial community in the given samples, conventional statistical analysis cannot be carried out in this study and data was subjected to evaluation as per earlier studies [3, 69, 78].The Mann-Whitney U test and the Wilcoxon W test was used to compare the abundance of the different species.

At the genus level, a circular phylogenetic maximum likelihood tree was constructed by using iTOL and PhyloT tools as per Letunic and Bork (**Letunic I, Bork P 2011)**[72].

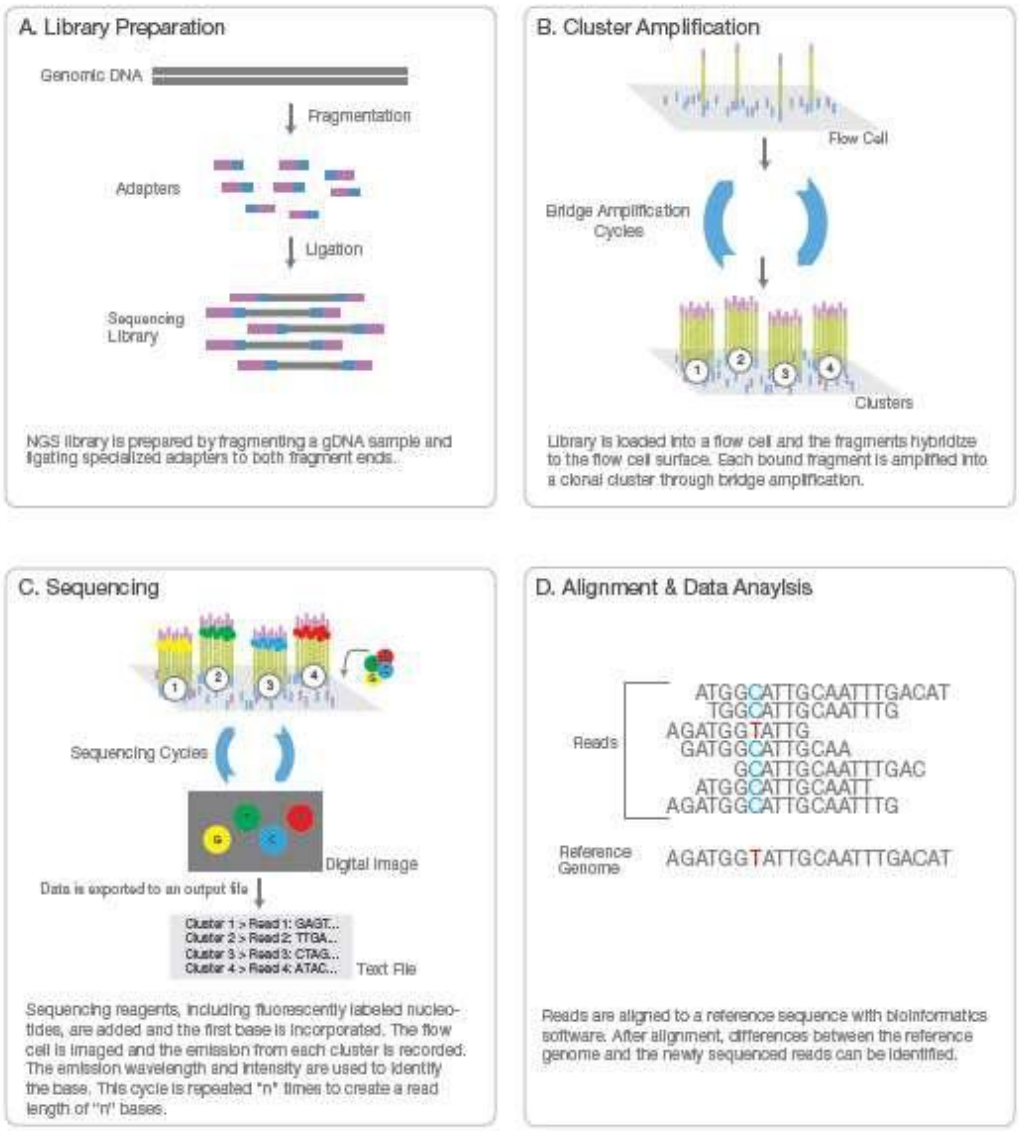
# *Photographs*

# SAMPLE COLLECTION

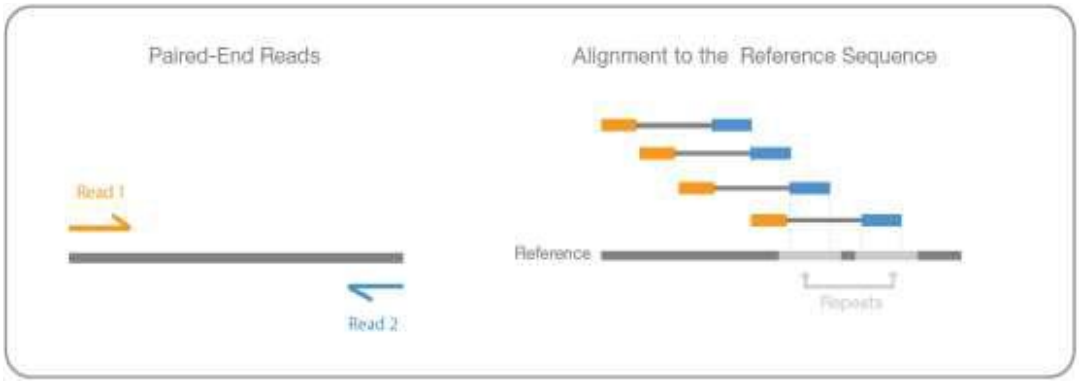


# ILLUMINA SEQUENCING

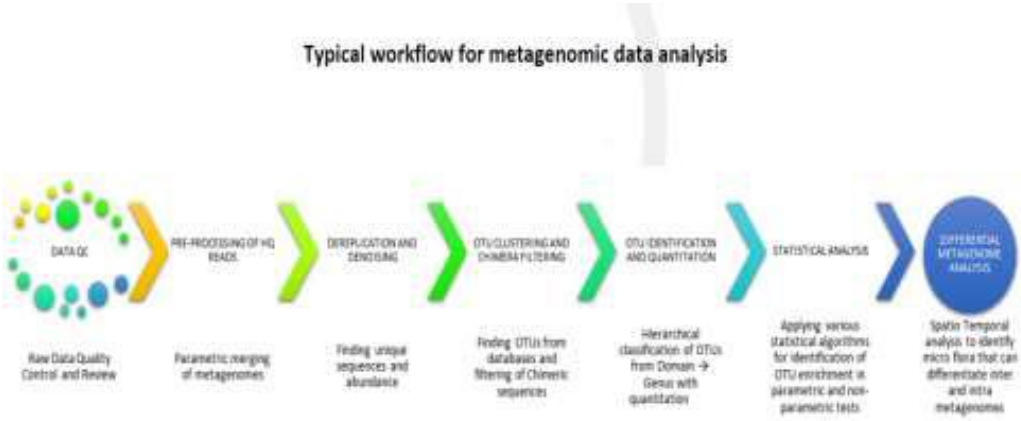
**4 basic steps in Illumina NGS work flow**



## Paired-End Sequencing and Alignment







# Results

# RESULTS

The study population comprised of 4 periodontally healthy individuals and 4 patients who had moderate to severe supragingival calculus, whose plaque samples were obtained and subjected to 16s rRNA sequencing using NGS technology.V3– V4 regions were sequenced and for each of the 8 plaque samples, bacterial phyla, genera and species were identified and their relative abundance quantified via taxonomic assignment against reference database.

**Periodontal Health/Control group:**

There were totally 7 phyla, 30 genera and 54 species found in the healthy samples.

In the healthy group, at the phylum level, Firmicutes were found to be more in abundance, followed by Proteobacteria and Candidatus Saccharibacteria which were about 24%, 22% and 18% respectively as shown in table 1-A and figure 1-A.

At the genus level (top 10), Veillonella was found to be more in abundance followed by Neisseria and Saccharibacteria genera incertae sedis which were about 16%, 14% 12% respectively as shown in table 2-A and figure 2-A.

At the species level (top 10), Veillonella tobetsuensis had more abundance followed by Neisseria subflava and Veillonella parvula which included 16%, 11% and 11% respectively as shown in table 3-A and figure 3-

A. Unclassified species accounted for about 14% among the top 10 species in health.

**Calculus/Test group:**

There were a total of 9 phyla, 30 genera and 52 species found in the calculus samples.

In the calculus group, at the phylum level, Firmicutes was found to be more in abundance of about 21% followed by Proteobacteria 19% and Candidatus Saccharibacteri 19% as shown in table 1-B and figure 1-B.

At the genus level (top 10), Veillonella, Saccharibacteria genera incertaesedis and Actinomyces had more abundance of about 14% 13% and 11% respectively as shown in table 2-B and figure 2-B.

At the species level (top 10), Unclassified species were the most predominant with an abundance of 14%, followed by Veillonella tobetsuensis 13% and Veillonella parvula 11% as shown in table 3-B and figure 3-B.

**Comparison of abundance of phyla between periodontal health and calculus group:**

The comparison of abundance of phyla between the health and the calculus group is represented in table 4-A and graph 1. There was more abundance of Firmicutes, proteobacteria in the healthy group as compared to the calculus/disease group. The abundance of Candidatus Saccharibacteria,

Actinobacteria, Synergistetes and Fusobacteria were found to be higher in the disease/calculus group as compared to the healthy group.

**Comparison of abundance of genera between periodontal health and disease/calculus group:**

The comparison of abundance of genera between the healthy and the calculus group is represented in the table 4-B and graph 2. Veillonella was the most abundant genera in both the groups. Neisseria, Haemophilus and Eikenella were higher in the healthy group than in the calculus group. Kingella was found exclusively in the healthy group. Dialister, Cardiobacterium and Capnocytophaga were present among the top 10 genera in the disease/calculus group, while its presence in the periodontally healthy group was not significant.

**Comparison of abundance of species between the periodontal health and calculus group:**

The comparison of abundance of top 17 species present in health vs calculus has been compared as shown in table 4-C and graph 3. Veillonella tobetsuensis, Unclassified and Neisseria subflava were found to be more abundant in the healthy group than in the calculus group. Haemophilus parainfluenzae, Eikenella corrodens, Lautropia mirabilis, Actinomyces meyeri and Fretibacterium fastidiosum were found to be more abundant in the healthy group when compared to the disease group. Aggregatibacter segnis, Dialister

invisus, Cardiobacterium hominis and Capnocytophaga leadbetteri were found to be more abundant in the disease/calculus group than in the healthy group.

**Statistical analysis of the comparison of overall abundance of periodontal health vs calculus group at the phylum, genus and species level:**

**Phylum Level:**

The mean with standard deviation for abundance of health group is 1.7969 ± 0.98941. The mean with standard deviation for abundance of disease/calculus group is 1.4019 ± 1.06107. The comparison of abundance of phyla between the healthy group and calculus group was not statistically significant at p=0.427 (P>0.05) as shown in graph 4.

**Genus Level:**

The mean with standard deviation for abundance in healthy group is 1.8788 ± 0.57003. The mean with standard deviation for abundance of disease/calculus group is 1.7880 ± 0.45555. The comparison of abundance of genus in health group and disease/calculus group was not statistically significant at p=0.833 (P>0.05) as shown in graph 4.

**Species Level:**

The mean with standard deviation for abundance of healthy group is 1.6299 ± 0.64327. The mean with standard deviation for abundance of disease/calculus group is 1.7358 ± 0.46607. The comparison of abundance of

species in health group and disease/calculus group was not found to be statistically significant at p=0.547 (P>0.05) as shown in graph 4.

**Comparison of microbiome in periodontal health versus calculus with circular maximum likelihood phylogenetic tree at genus level:**

The microbiome was compared between periodontally healthy and calculus group. At the genus level, a circular phylogenetic maximum likelihood tree (figure 4) was constructed by using iTOL and PhyloT tools as per Letunic and Bork (**Letunic I, Bork P 2011)**[72]**.**The bars in the outer band (green) represent the relative abundance of bacterial genera in healthy (orange) and calculus (blue) sites.

# *Tables & Graphs*

# TABLES & GRAPHS

**Table 1-A: Evaluation of abundance of phyla and their percentage among the Periodontal Health samples**

| S.NO | Phylum | Relative abundance expressed in logs | Percentage of phyla |
|---|---|---|---|
| **1.** | Firmicutes | 3.06 | 24% |
| **2.** | Proteobacteria | 2.80 | 22% |
| **3.** | Candidatus Saccharibacteria | 2.21 | 18% |
| **4.** | Actinobacteria | 1.75 | 14% |
| **5.** | Bacteroidetes | 1.56 | 12% |
| **6.** | Synergistetes | 0.90 | 7% |
| **7.** | Fusobacteria | 0.30 | 3% |

**Figure 1-A: Comparison of percentage of phyla in the Periodontal Health group**

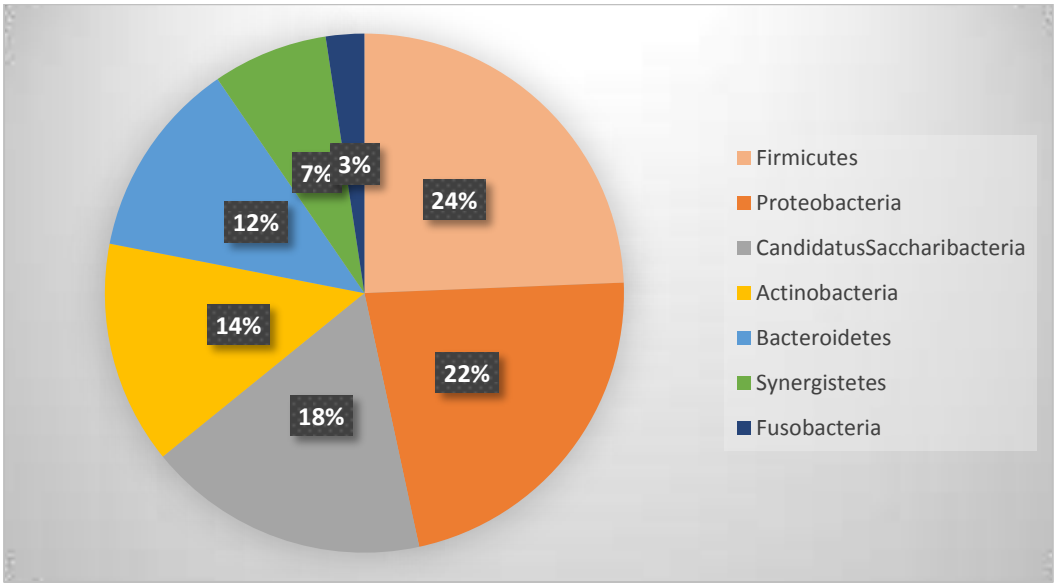


**Table 1-B: Evaluation of abundance of Phyla and their percentage among calculus samples**

| S.No | Phylum | Relative abundance expressed in logs | Percentage of Phyla |
|---|---|---|---|
| **1.** | Firmicutes | 2.67 | 21% |
| **2.** | Proteobacteria | 2.41 | 19% |
| **3.** | Candidatus Saccharibacteria | 2.38 | 19% |
| **4.** | Actinobacteria | 2.08 | 17% |
| **5.** | Bacteroidetes | 1.54 | 12% |
| **6.** | Synergistetes | 1.04 | 8% |
| **7.** | Fusobacteria | 0.48 | 4% |

**Figure 1-B: Comparison of percentage of phyla in the Calculus group**

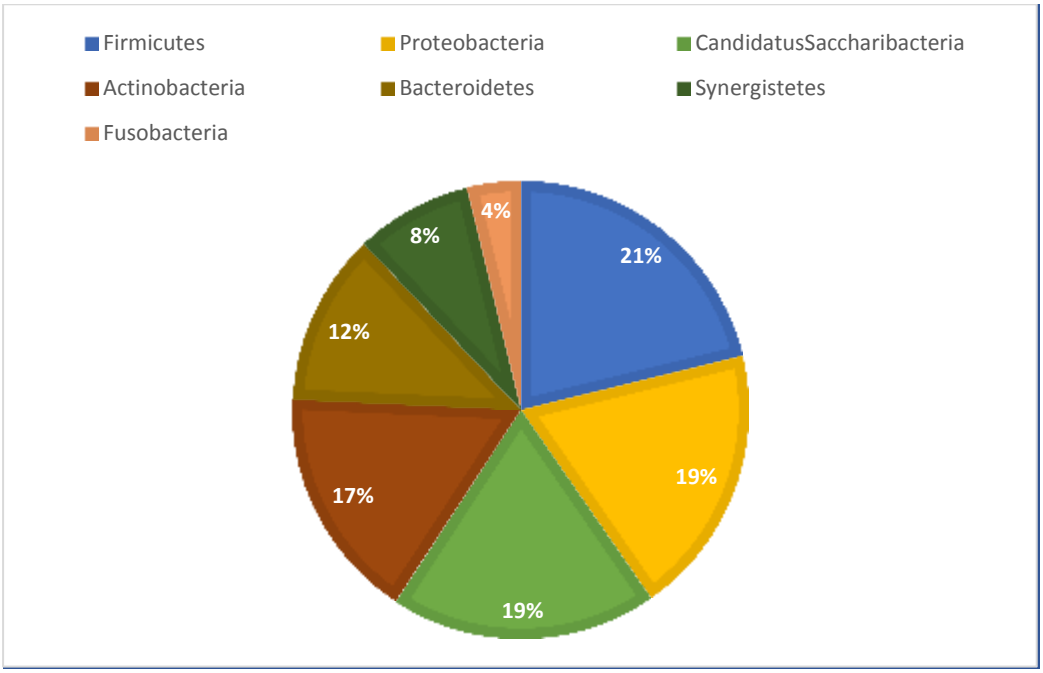


**Table 2-A: Evaluation of abundance of top 10 Genera and their percentage among the Periodontal Health samples**

| S.NO | Genus | Relative Abundance expressed in logs | Percentage of Genera |
|---|---|---|---|
| **1.** | Veillonella | 3.05 | 16% |
| **2.** | Neisseria | 2.63 | 14% |
| **3.** | Saccharibacteria genera incertaesedis | 2.21 | 12% |
| **4.** | Haemophilus | 1.84 | 10% |
| **5.** | Actinomyces | 1.71 | 9% |
| **6.** | Kingella | 1.59 | 8% |
| **7.** | Aggregatibacter | 1.48 | 8% |
| **8.** | Eikenella | 1.46 | 8% |
| **9.** | Lautropia | 1.45 | 8% |
| **10.** | Prevotella | 1.38 | 7% |

**Figure 2-A: Comparison of percentage of top 10 genera in the Periodontal Health group**

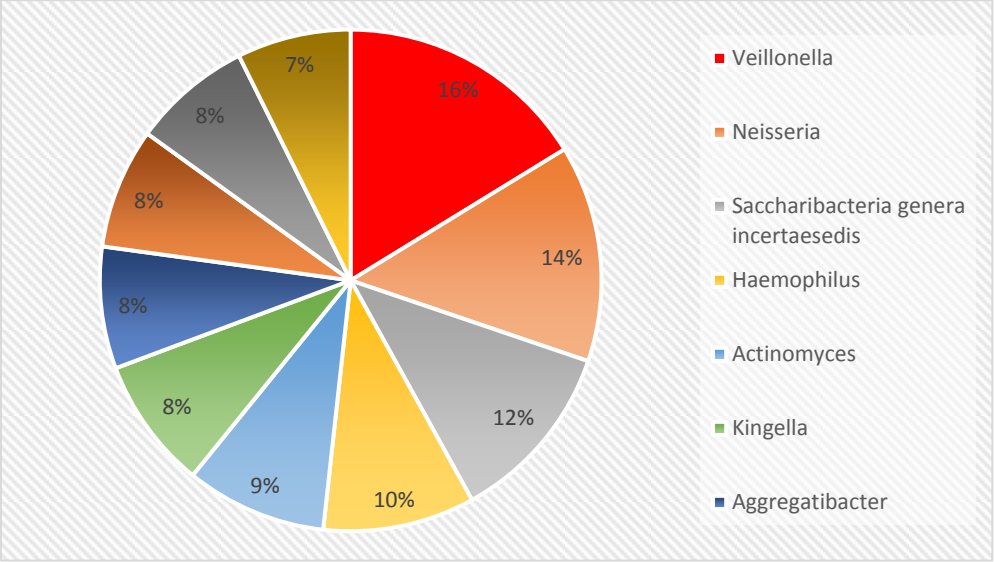


**Table 2-B: Evaluation of abundance of top 10 Genera and their percentage among calculus samples**

| S.NO | GENUS | Relative abundance expressed in logs | Percentage of genera |
|---|---|---|---|
| **1.** | Veillonella | 2.57 | 14% |
| **2.** | Saccharibacteria genera incertaesedis | 2.38 | 13% |
| **3.** | Actinomyces | 2.08 | 11% |
| **4.** | Aggregatibacter | 1.92 | 10% |
| **5.** | Neisseria | 1.90 | 10% |
| **6.** | Dialister | 1.81 | 10% |
| **7.** | Cardiobacterium | 1.70 | 9% |
| **8.** | Haemophilus | 1.57 | 9% |
| **9.** | Capnocytophaga | 1.36 | 7% |
| **10.** | Solobacterium | 1.34 | 7% |

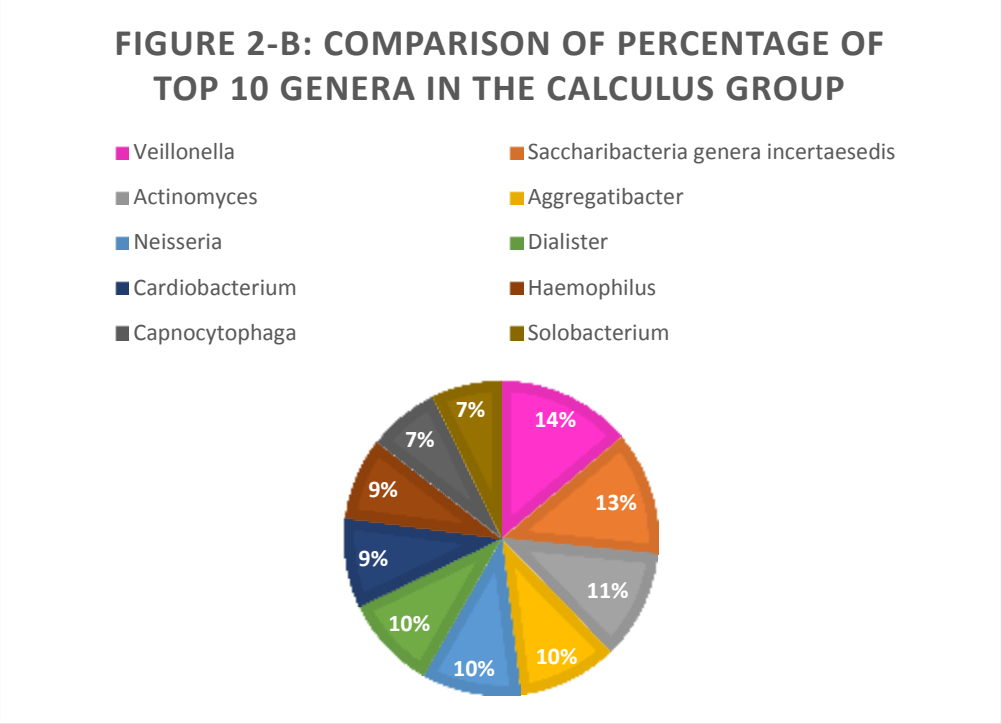


**Table 3-A: Evaluation of abundance of top 10 Species and their percentage among Periodontal Health samples**

| S.No | Species | Relative abundance expressed in logs | Percentage of Species |
|---|---|---|---|
| **1.** | Veillonella tobetsuensis | 3.01 | 16% |
| **2.** | Unclassified | 2.67 | 14% |
| **3.** | Neisseria subflava | 2.06 | 11% |
| **4.** | Veillonella parvula | 2.00 | 11% |
| **5.** | Haemophilus parainfluenzae | 1.81 | 9% |
| **6.** | Kingella oralis | 1.59 | 8% |
| **7.** | Actinomyces odontolyticus | 1.58 | 8% |
| **8.** | Eikenella corrodens | 1.46 | 8% |
| **9.** | Lautropia mirabilis | 1.45 | 8% |
| **10.** | Aggregatibacter segnis | 1.34 | 7% |

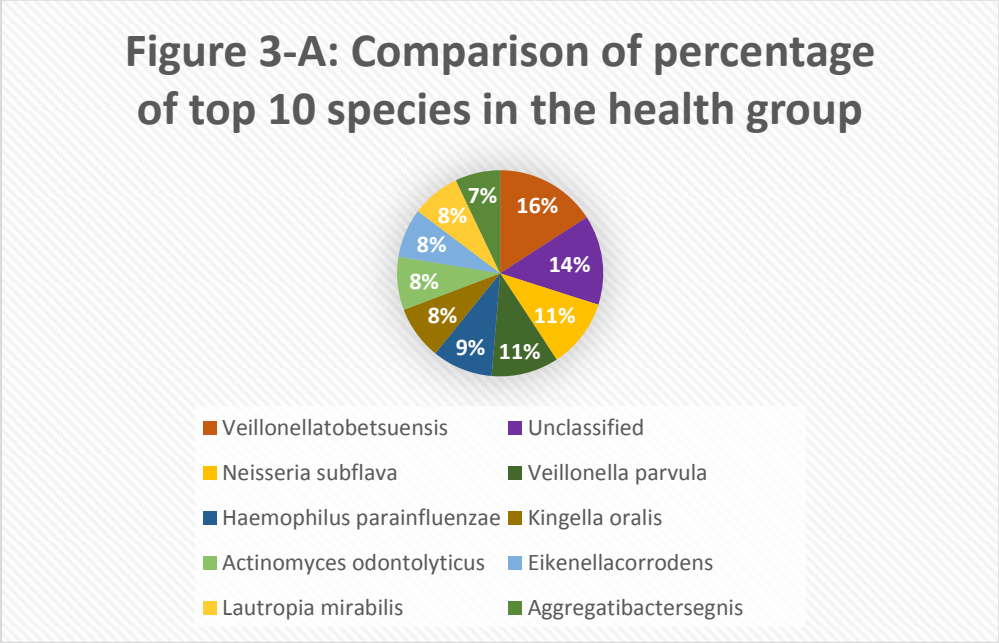


**Table 3-B: Evaluation of abundance of top 10 Species and their percentage among calculus samples**

| S.NO | Species | Relative abundance expressed in logs |
|---|---|---|
| **1.** | Unclassified | 2.48 |
| **2.** | Veillonella tobetsuensis | 2.37 |
| **3.** | Veillonella parvula | 2.06 |
| **4.** | Actinomyces haliotis | 2.00 |
| **5.** | Aggregatibacter segnis | 1.86 |
| **6.** | Dialister invisus | 1.80 |
| **7.** | Cardiobacterium hominis | 1.57 |
| **8.** | Solobacterium moorei | 1.34 |
| **9.** | Neisseria subflava | 1.30 |
| **10.** | Veillonella denticariosi | 1.18 |

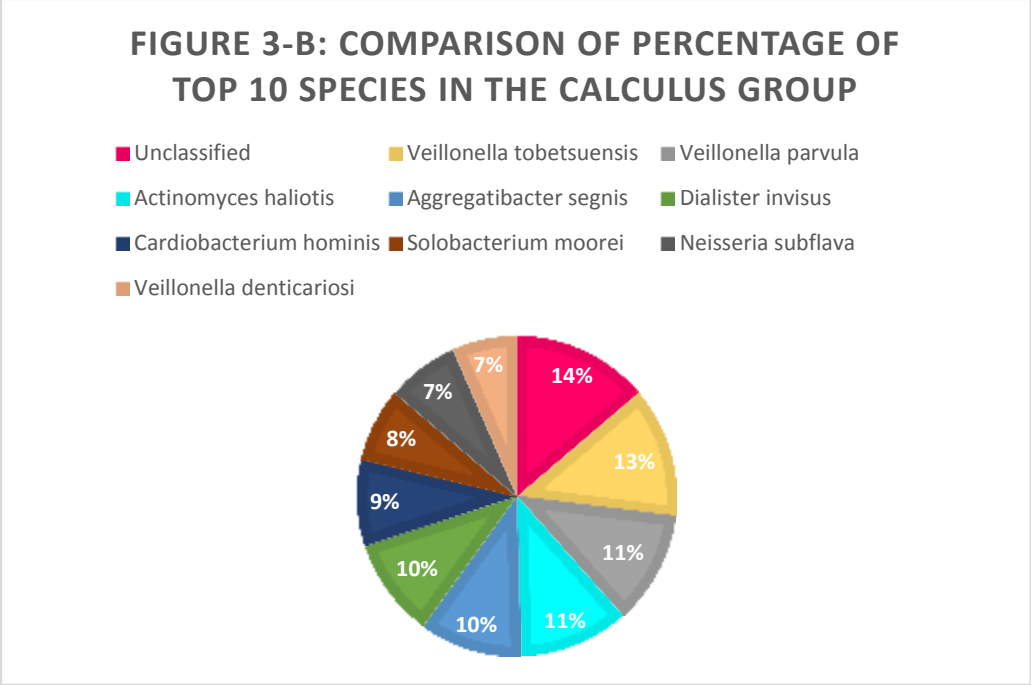


**Table 4-A: Comparison of abundance of phylum in Periodontal Health and calculus**

| S.NO | Organisms | Periodontal Health | Calculus |
|---|---|---|---|
| **1.** | Firmicutes | 3.06 | 2.67 |
| **2.** | Proteobacteria | 2.80 | 2.41 |
| **3.** | Candidatus saccharibacteria | 2.21 | 2.38 |
| **4.** | Actinobacteria | 1.75 | 2.08 |
| **5.** | Bacteroidetes | 1.56 | 1.54 |
| **6.** | Synergistetes | 0.90 | 1.04 |
| **7.** | Fusobacteria | 0.30 | 0.48 |

**Graph 1: Comparison of abundance of phylum in Periodontal Health and calculus**

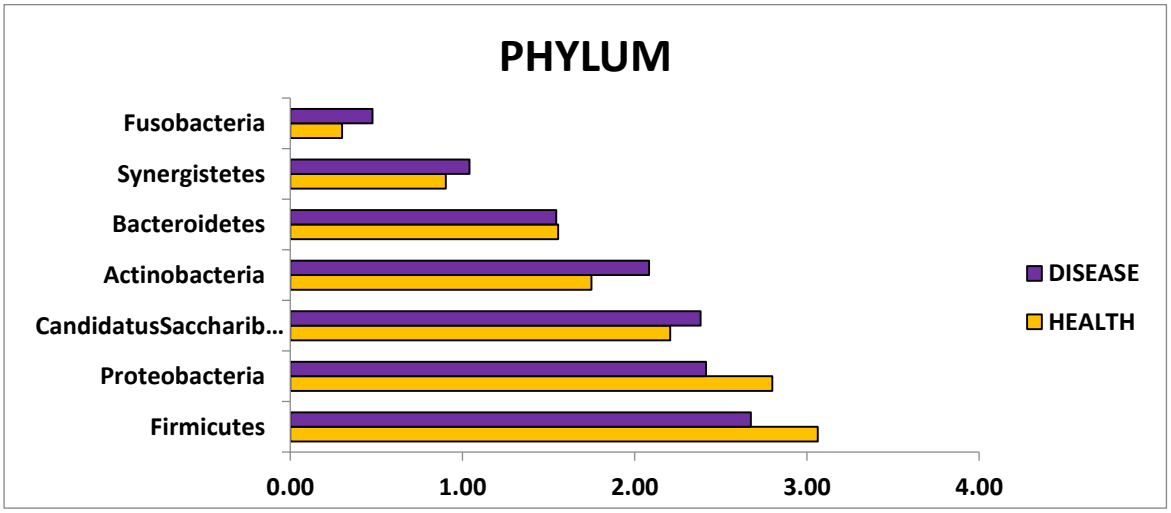
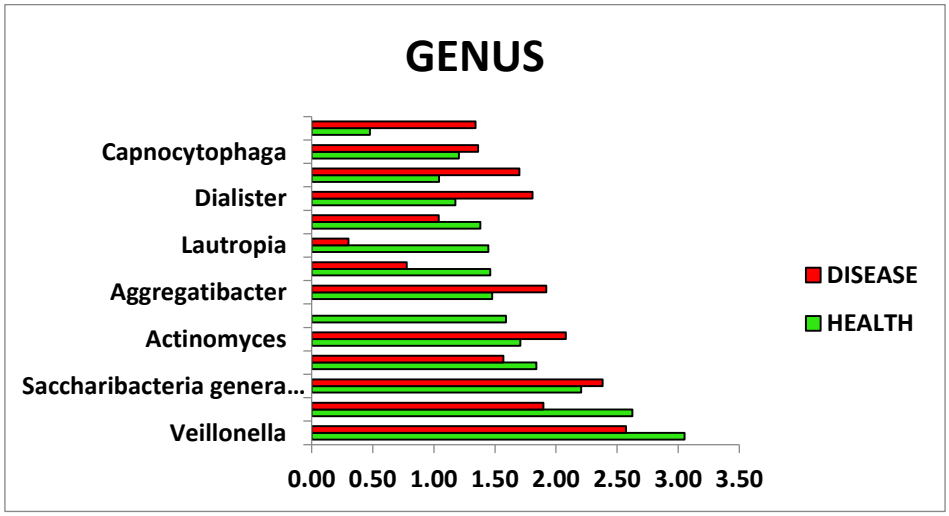


**Table 4 B: Comparison of abundance of genus in Periodontal Health and calculus**

| S.No | Organisms | Periodontal Health | Calculus |
|---|---|---|---|
| **1.** | Veillonella | 3.05 | 2.57 |
| **2.** | Neisseria | 2.63 | 1.90 |
| **3.** | Saccharibacteria genera incertaesedis | 2.21 | 2.38 |
| **4.** | Haemophilus | 1.84 | 1.57 |
| **5.** | Actinomyces | 1.71 | 2.08 |
| **6.** | Kingella | 1.59 | 0.00 |
| **7.** | Aggregatibacter | 1.48 | 1.92 |
| **8.** | Eikenella | 1.46 | 0.78 |
| **9.** | Lautropia | 1.45 | 0.30 |
| **10.** | Prevotella | 1.38 | 1.04 |
| **11.** | Dialister | 1.18 | 1.81 |
| **12.** | Cardiobacterium | 1.04 | 1.70 |
| **13.** | Capnocytophaga | 1.20 | 1.36 |
| **14.** | Solobacterium | 0.48 | 1.34 |

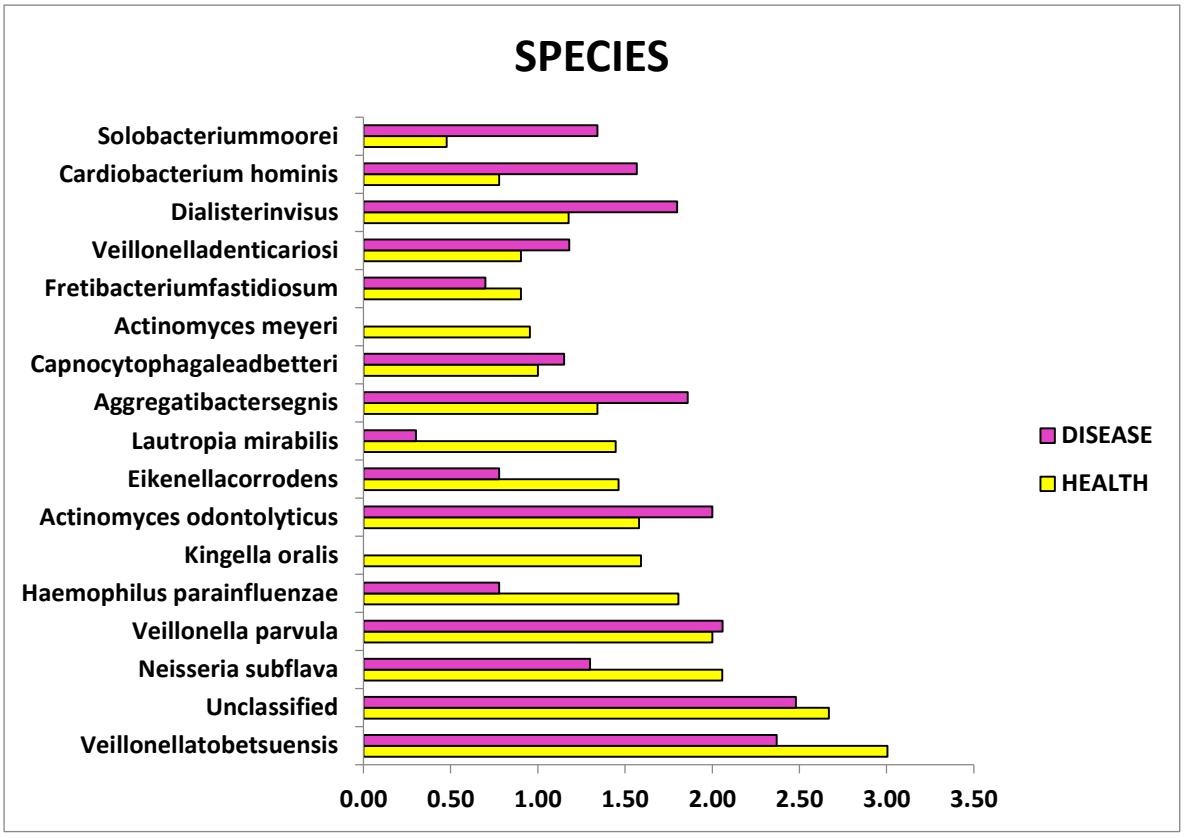
**Graph 2 – Comparison of abundance of genus in Periodontal Health and calculus**



**4-C: Comparison of abundance of species in Periodontal Health and calculus**

| S.NO | Organisms | Periodontal Health | Calculus |
|---|---|---|---|
| **1.** | Veillonella tobetsuensis | 3.01 | 2.37 |
| **2.** | Unclassified | 2.67 | 2.48 |
| **3.** | Neisseria subflava | 2.06 | 1.30 |
| **4.** | Veillonella parvula | 2.00 | 2.06 |
| **5.** | Haemophilus parainfluenzae | 1.81 | 0.78 |
| **6.** | Kingella oralis | 1.59 | 0.00 |
| **7.** | Actinomyces odontolyticus | 1.58 | 2.00 |
| **8.** | Eikenella corrodens | 1.46 | 0.78 |
| **9.** | Lautropia mirabilis | 1.45 | 0.30 |
| **10.** | Aggregatibacter segnis | 1.34 | 1.86 |
| **11.** | Capnocytophaga leadbetteri | 1.00 | 1.15 |
| **12.** | Actinomyces meyeri | 0.95 | 0.00 |
| **13.** | Fretibacterium fastidiosum | 0.90 | 0.70 |
| **14.** | Veillonella denticariosi | 0.90 | 1.18 |
| **15.** | Dialister invisus | 1.18 | 1.80 |
| **16.** | Cardiobacterium hominis | 0.78 | 1.57 |
| **17.** | Solobacterium moorei | 0.48 | 1.34 |

**Graph 3 - Comparison of abundance of species in Periodontal Health and calculus**



**Graph 4 - Statistical Analysis of the Comparison of abundance of the Periodontal Health vs calculus given at the Phylum, Genus and Species level:**

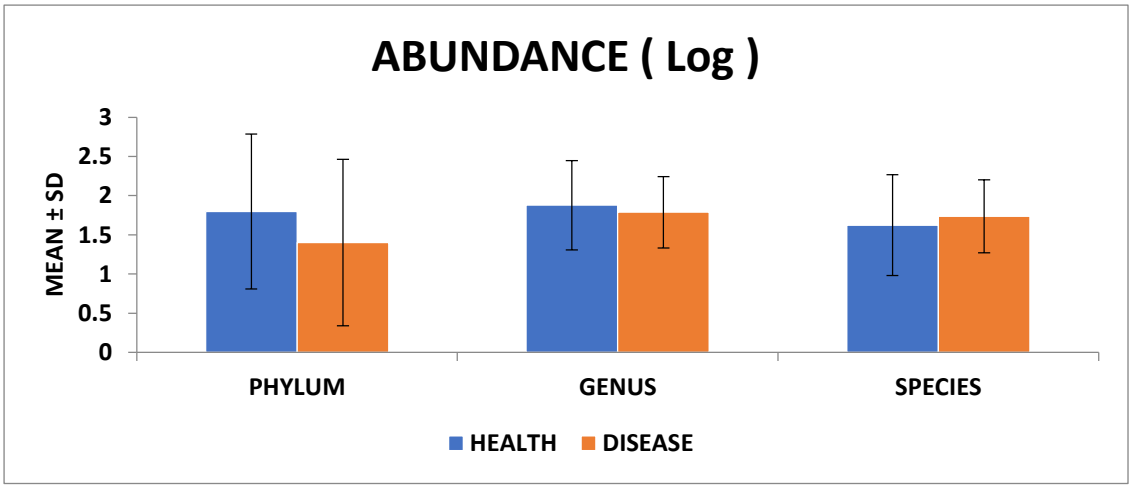
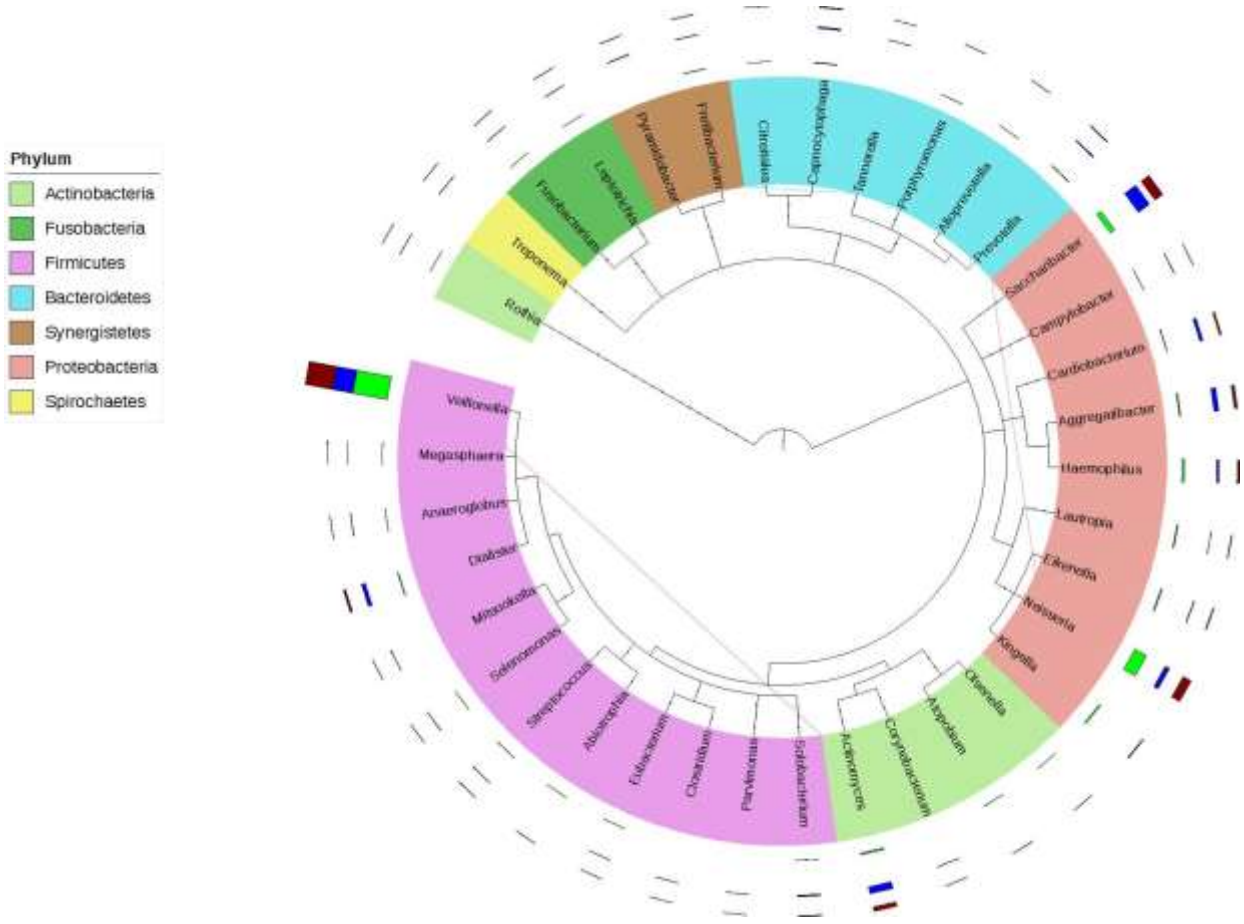


**Figure 4: Comparison of microbiome in Periodontal Health versus calculus with circular maximum likelihood phylogenetic tree at genus level:**



# *Discussion*

# DISCUSSION

Periodontal disease is a chronic inflammatory disease resulting from an interplay of bacterial challenge and the host response which results in the loss of the attachment apparatus of the tooth. The disease is known to be modified by environmental, acquired risk factors and genetic susceptibility[105].The oral cavity represents one of the most diverse microbial communities and is highly complex with around 700 species identified in different ecological niches in the oral cavity[112].

The diversityof bacterial species in the subgingival flora, the variation in composition of floras from individual to individual and the variation in host response to bacterial species are some of the major reasons that the specific etiology of periodontal disease has not been clearly established[136]. Some of the most common organisms associated with periodontal diseases are Porphyromonas gingivalis, Prevotella intermedia, Tannerellaforysythia, Aggregatibacteractinomycetemcomitans, as well as the treponemes[124].

Dental plaque represents a classic example of both a biofilm and a microbial community, in that it displays emergent properties, i.e., plaque displays properties that are more than the sum of its constituent members, and microbial communities are ubiquitous in nature and usually exist attached to a surface as a spatially organized biofilm[124, 92]. Recent studies suggest that the environmental heterogeneity generated within biofilm promotes accelerated genotypic and phenotypic diversity that provides a form of “biological

insurance" that can safeguard the microbial community in the face of adverse conditions, survive the host response and cause the disease[92].

The correlation between the plaque mass and pathological changes was put forth as the non-specific plaque theory of periodontal disease. It assumes that the qualitative features of the microbiota are of minor importance and that the mass rather than the composition of the plaque is crucial to the pathogenesis of the disease[77]. But the association of Aggregatibacter actinomycetemcomitans with localized aggressive periodontitis could not be explained by the above hypothesis.

To explain the above observation, the specific plaque hypothesis was put forth by **Loesche**[83] who stated that not all organisms or plaque as a whole was involved in the pathogenesis of periodontal disease, but only specific microorganisms were associated with it. To address the inadequacies with the earlier hypotheses, Marsh proposed the ecological plaque hypothesis in which he attributed the environmental perturbations and the resulting imbalance in the subgingival microflora to enhance the pathogenicity and thus cause the disease[92].

The present understanding is that periodontal disease is initiated by an environment/ ecosystem where synergistic microbiota converges to form a disease provoking one resulting in dysbiosis. The whole bacterial community may be health or disease associated[45] .

Dental calculus is nothing but mineralized dental plaque composed primarily of calcium phosphate mineral salts which is deposited on natural teeth and restorations and is covered by a layer of unmineralized plaque[152, 153].

The rough calculus surface may not, in itself, induce inflammation in the adjacent periodontal tissues, instead it serves as an ideal substrate for subgingival microbial colonization[57] and also

• acts as a niche which harbors bacterial plaque

• acts as an irritant to the periodontal tissues

• distends the periodontal pocket wall

• inhibits the ingress of polymorphonuclear leukocytes.

Supragingival and subgingival calculus progressing in an apical direction or laterally is directly responsible for the pocket deepening and loss of attachment[150,152], as a metabolically active biofilm always covers subgingival calculus. The clinical appearance of the periodontal tissues may be affected by subgingival dental calculus because it provides a rough surface for retention and establishment of microorganisms and hence indirectly impairs sufficient removal of biofilm, the cause of periodontal disease. Subgingival calculus is also a by-product, which owes its secondary development to the presence of microorganisms and exudative inflammatory products[15].

A variety of techniques for analyzing the plaque samples have been developed in the past which include microscopy, bacterial culture, enzymatic assays, immunoassays, nucleic acid probes and polymerase chain reaction assays[113], and yet faced difficulties with identification and evaluation of the pattern of the microbial diversity in the periodontal environment. In the recent years, sequencing of the 16srRNA gene has been widely used to study the microbial profile and to characterize the shifts that occur from periodontal health to disease[69]. The open-ended methods viz. Sanger and Pyrosequencing were used but were found to be laborious, time consuming and expensive[93].

Currently, the NGS technology offers great promise in the study of the metagenome as it is a high throughput, deep sequencing method which is economical and has the ability to quantify the abundance of bacterial species[13, 132], has been applied in this study for sequencing microbiome from plaque samples that was present on the surface of supragingival calculus and was compared with the microbial profile of plaque in periodontal health.

In this study, plaque that was covering supragingival calculus was obtained from 4 individuals who had moderate to severe calculus and inflammation and supragingival plaque from 4 periodontally healthy subjects using sterile gracey's curette. The samples were then eluted in an Eppendorf tube containing molecular grade ionized water and was sent for microbial analysis.

Genomic DNA was extracted from all the samples using the Fast DNA kit and Fast Prep 24 5G instrument as per the manufacturer's protocols. Extracted DNA was then amplified using the 16sV3 forward and V4 reverse primer pairs with added Illumina adapter overhang nucleotide sequences. The study was carried out in the Illumina Solexa sequencer where in more sequence per run could be obtained and low abundance taxa determined at a relatively lesser cost[8].

The results of the study showed the presence of 7 phyla, 30 genera and 54 species in the periodontally healthy group as against 9 phyla, 30 genera and 52 species in the calculus group. Though there was an increase in the number of phyla in the calculus group, there was no difference in the number of genera and a slight decrease in the species in the calculus group.

Though Firmicutes and Proteobacteria were the most abundant phyla present in both the groups, there was a marginal increase in their abundance in the calculus group when compared to the healthy group. The other phyla present in both the groups were Candidatus saccharibacteria, Actinobacteria, Synergistetes and Fusobacteria which were marginally higher in the calculus group. There was no statistically significant difference ($p=0.427$, $p>0.05$) in the overall abundance of the phyla in the calculus group when compared to the periodontally healthy group.

So, at the phyla level, there was a highly similar microbial profile in both the groups and their presence are in line with the previous studies of **Kistler et al. [64], Park et al**.[111], but different from the study of **Griffen et al.**[3].

This could be due to the fact that in this study the plaque samples were collected using curettes as against the paper points used in the study by **Griffen**[3]. When paper points are used, it only allows passive diffusion of the plaque material and hence could lead to under sampling and represent only the outer biofilm microorganisms. These phyla are known to comprise of species that are the predominant members of the primary colonizers of gram -positive cocci.

At the genus level, Veillonella was the most abundant genus in both the groups but slightly higher in abundance in the healthy group (16%) when compared to the calculus group (14%) followed by Neiserria and Heamophilus. Veillonella, a small gram- negative cocci is a known primary colonizer in the periodontal environment which facilitates species succession in the biofilm community. The marginal increase in the abundance of Heamophilus in health when compared to the calculus group is in accordance with the study of **Camelo Castillo et al.**[13], but differs from the study of **Park et al.**[111]who has reported the association of this organism, along with streptococcus in the development of gingivitis. But surprisingly, in this study, streptococcus was not present among the top 15 abundant genera in either of the groups. This could be due to the ecological perturbations caused by factors viz. dietary patterns and lifestyle changes which could have impacted the biofilm composition in this population[84].

Kingella, a member of the HACEK groupwas found to be present exclusively in the healthy group. All the other members of the HACEK group viz. Eikenella and Aggregatibacter[21]were also found in the top ten abundant genera in the healthy group. Cardiobacterium was found in both the groups, with its abundance being more in the calculus group. The acronym HACEK refers to a group of fastidious gram-negative cocco bacillary organisms that colonize the oropharyngeal region and have been associated with endocarditis[128]. The presence of these organisms in the periodontal environment has been demonstrated in previous studies by **Colombo et al.**[22], and also in an earlier study carried out in our department (unpublished data) which suggest that these could be a part of the core microbiome in the periodontal milieu.

There was an increase in the abundance of the genus Dialister in the calculus group than in the healthy group where it was present only in small numbers. Dialister is a gram negative, obligately anerobic, non-motile coccobacilli which occur singly, in pairs or in short chains making them difficult to identify in mixed cultures and might require molecular methods for identification[98]. Recently, the role of certain phylotypes of this genus has been established in the pathogenesis of periodontal disease[110]and their very presence in the calculus group is suggestive of their involvement in the shift of the ecosystem to a disease associated one. The slight increase in the abundance of the genus Capnocytophaga in the calculus group further

reiterates the fact that the biofilm as a whole rather than individual bacteria contribute to the pathogenesis of the disease.

At the species level, Veillonella tobetsuensis is the most abundant species in the healthy group and the second most abundant one next only to unclassified species in the calculus group. Veillonella parvula is the other predominant species in both the groups suggestive of the fact that that the presence of these early colonizers is a requisite for the initial growth of the biofilm, irrespective of its association with health or disease[54]. The increased abundance of Dialister invisus and Aggregatibacter segnis in the calculus group seems to suggest a pathogenic role of these organisms in the progression of the disease process, seen predominantly as attachment loss with gingival recession in our population. But there was no statistically significant difference in the overall abundance of the top 15 species in between the groups.

With the microbial profile/pattern obtained in this study, a distinct microbial profile in the biofilm that covers the calculus could not be definitely established. It could be due to the fact that there is similarity in the environment form where the plaque has been obtained viz. the oxygen stress levels, the pH and the carbohydrate availability. Hence, a concrete microbial pattern is not evidently shown. However, there are some interesting differences that could be possibly explored in a larger sample size and in a more longitudinal manner, right from the formation of the supragingival

biofilm on the tooth surface to its mineralization and the formation of a biofilm on the well-formed calculus surface, which could contribute to the predominant pattern of periodontal disease (Attachment loss associated with gingival recession) that is observed in this part of the globe[87].

The major limitation of the study is the small sample size used which comprised of only four subjects in each group, due to the complexity of the technique and the forbidding cost. Nonetheless, the advent of the newer molecular approaches and the use of NGS technology in the study of the microbial profile/community have immensely widened the horizons in the understanding of the pathogenesis of the disease process.

# *Summary & Conclusion*

# SUMMARY AND CONCLUSION

The rationale for the study was to evaluate the microbial profile of the plaque biofilm that covers the supragingival calculus and to compare it with that of periodontal health by 16s rRNA sequencing method with the use of NGS technology. Due to the complexity of the technique involved and its prohibitory cost, a total of 8 samples were evaluated which included 4 periodontally healthy individuals and 4 patients who had presence of moderate to severe calculus and gingiva inflammation. The plaque samples were collected using sterile gracey's curette, placed in molecular grade ionized water and sent for further analysis.

The genomic DNA was extracted, 16s rRNA amplification, library construction and sequencing were done and data was interpreted. The results of the study showed the presence of 7 phyla, 30 genera and 54 species in the periodontally healthy group and 9 phyla, 30 genera and 52 species in the calculus group. At the phyla level, there was a very similar microbial pattern in both the groups. At the genus level, Veillonella was the most abundant genus in both the groups, along with other genera viz. Heamophilus and Neisseria. Kingella, a member of the HACEK group was found exclusively in the healthy group. The other members of the HACEK group were also present, but their role in the pathogenesis is not yet established. The increased presence of newer periodontal pathogens viz. Dialister in the calculus group suggests

that there could be a possible shift in the ecosystem which could contribute to the progression of the disease.

At the species level, there was a similar microbial pattern in both the groups, except for the elevated presence of Dialister invisus and Aggregatibacter segnis in the calculus group. But there was no statistically significant difference in the overall abundance of the species in between the groups. The results of the study did not identify a distinct microbial profile, yet points to a microbial shift that could contribute to the progression of the disease process that is characteristically seen in our population, that which is associated with gingival recession.

To conclude, the microbiome of the plaque biofilm that covers calculus warrants future exploration with a larger sample size and in a longitudinal manner to draw meaningful clinical implications.

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# *Annexures*

# ANNEXURE –I

**RAGAS DENTAL COLLEGE & HOSPITAL**
(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai
2/102, East Coast Road, Uthandi, Chennai - 600 119. INDIA.
Tele : (044) 24530002, 24530003-06. Principal (Dir) 24530001 Fax : (044) 24530009

**TO WHOMSOEVER IT MAY CONCERN**

DATE: 04.2.2019

Chennai.

From

The Institutional Review Board,

Ragas Dental College and Hospital,

Uthandi, Chennai- 600119.

The Dissertation topic titled **"EVALUATION OF MICROBIOME IN PERIODONTAL HEALTH AND PLAQUE COVERING SUPRAGINGIVAL CALCULUS USING NEXT GENERATION SEQUENCING TECHNOLOGY"** Submitted by **DR. SANTHOSH KUMAR** has been approved by the Institutional Review Board of Ragas Dental College & Hospital.

**Dr.N.S.Azhagarasan, M.D.S**
Member Secretary,
Institutional Ethical Board,
Ragas Dental College and Hospital,
Uthandi, Chennai - 600119

# ANNEXURE –II

URKUND

## Urkund Analysis Result

| | |
|---|---|
| **Analysed Document:** | Plagirism check 2 (1).docx (D47718899) |
| **Submitted:** | 2/8/2019 5:14:00 AM |
| **Submitted By:** | ksanthosh_100@yahoo.co.in |
| **Significance:** | 6 % |

### Sources included in the report:

doc for analysis.docx (D34901232)
https://quizlet.com/114706733/plaque-formation-flash-cards/
https://patents.google.com/patent/US20140335534A1/en
https://patents.justia.com/patent/20160055296
https://pdfs.semanticscholar.org/4c5d/19d49097381ea0e715b15a7f1a12ca86ff87.pdf
http://dentistry-papers.blogspot.com/2010/

### Instances where selected sources appear:

28

**ANNEXURE III**

**CONSENT FORM**

I …… S/o, w/o, d/o……

aged about ……. years, Hindu/Christian/Muslim …… ……. residing at ……do solemnly

And state as follows.

I am the deponent herein; as such I am aware of the facts stated here under

I state that I came to Ragas Dental College and Hospital, Chennai for my treatment for

……

……

I was examined by Dr……..and I was requested to do the following

1. Full mouth Plaque Score

2. Full mouth bleeding score

3 Measurement of periodontal pocket depth and clinical attachment loss

I was also informed and explained about the collection of plaque during scaling in ……(language) known to me. I was also informed and explained that the results of the individual test will not be revealed to the public. I give my consent after knowing full consequence of the dissertation/thesis/study and I undertake to cooperate with
the doctor for the study.

I also authorize the Doctor to proceed with further treatment or any other suitable alternative method for the study,

I have given voluntary consent to the collection of plaque for approved research.

I am also aware that I am free to withdraw the consent given at any time during the study in writing.

Signature of the patient/Attendant

The patient was explained the procedure by me and has understood the same and with full consent signed in (English/Tamil/Hindi/Telugu?.....) before me

Signature of the Doctor